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"TOXICOLOGICAL EVALUATION OF THE NEUROTOXIC PROPERTIES OF CYLINDROSPERMOPSIN AND ITS POTENTIAL INTERACTIONS WITH CYANOTOXINS AND CHEMICAL POLLUTANTS PRESENT IN FOOD"

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I. SUMMARY

SUMMARY

Cyanobacteria are prokaryotic microorganism present in many aquatic and terrestrial environmental samples worldwide able to produce some toxic compounds known as cyanotoxins. Among them, microcystins, in special microcystin-LR (MC-LR) and CYN are some of the most common ones. Both are produced by several species of cyanobacteria.

Cylindrospermopsin (CYN) is a cyanotoxin produced by several species of cyanobacteria. Their incidence is increasing in the last decades due to anthropogenic activities and climate change. These hepatotoxin and cytotoxin, respectively, are able to cause intoxication cases in the food chain from fish to humans, as they can also be bioaccumulated. The main mechanisms of action for MC-LR are the the inhibition of protein phosphatases, oxidative stress generation and effects on DNA repair, among others. Furthermore, MC-LR has demonstrated to exert neurotoxicity by irreversible inhibition of Serine/Threonine protein phosphatases 1 and 2A (PP1 and PP2A), Tau hyperphosphorylation, oxidative stress and modifications in neurotransmitters such as ACh, DA and GABA.

Concerning the mechanisms of action for CYN, the most important ones are the protein-synthesis inhibition, oxidative stress generation and apoptosis. Furthermore, its metabolite has demonstrated also to be able to cause genotoxicity. Despite being the liver and the kidneys its main targets, the nervous system has recently been studied as a possible target as well. The studies performed in this regard point out a possible effect on the production of oxidative stress and alterations in the acetylcholinesterase activity both *in vitro* and *in vivo*. However, the studies in this aspect are scarce.

Nonetheless, these cyanotoxins are not usually found in nature as a pure compound, but in combination with other chemicals such as other cyanotoxins. Taking into account all this information, a state of the art of the neurotoxicity induced by these cyanotoxins was perfomed, which led to the publication:

NEUROTOXICITY INDUCED BY MICROCYSTINS AND CYLINDROSPERMOPSIN: A REVIEW *(Hinojosa et al., 2019. Science of the Total Environment 668, 547-565)*

According to EFSA, it is important to study their toxicity not only isolated but also in combination, as they could be found in nature, due to their possible interaction, which could lead to changes in their effect. In order to assess the toxicological properties that these cyanotoxins would exert in a human cell line, both isolated and in combination, some studies concerning cytotoxicity, oxidative stress, acetylcholinesterase activity, and morphological analysis were

performed in the undifferentiated and differentiated SH-SY5Y human neuroblastoma cell line, which led to the following publication:

NEUROTOXIC ASSESSMENT OF MICROCYSTIN-LR, CYLINDROSPERMOPSIN AND THEIR COMBINATION ON THE HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE *(Hinojosa et al., 2019. Chemosphere 224, 751-764)*

Furthermore, taking into account that the studies concerning the neurotoxicity of CYN are scarce, the cytotoxic effects of a cyanobacterial extract containing CYN were observed in the undifferentiated SH-SY5Y in order to clarify if the concomitance with some other cyanobacterial compounds would produce changes compared to the effects observed by CYN itself, leading to the following publication:

CYTOTOXIC EFFECTS AND OXIDATIVE STRESS PRODUCED BY A CYLINDROSPERMOPSIN PRODUCING EXTRACT VERSUS CYLINDROSPERMOPSIN NON-PRODUCING EXTRACT ON THE HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE *(Hinojosa et al., 2021. Environmental Toxicology. In preparation)*

As CYN demonstrated to cause damage on the neuroblastoma cell line, the effects of this cyanotoxin were studied in the embryonic murine primary neurons, by studying cytotoxicity and synaptic biomarkers, which led to the following manuscript:

EFFECTS OF CYLINDROSPERMOPSIN ON CYTOTOXICITY AND NEUROTRANSMISSION ON MURINE PRIMARY CULTURES OF NEURONS *(Hinojosa et al., 2021. In preparation)*

On the other hand, some of the most common pollutants found in aquatic ecosystems are pesticides, being organophosphates an important percentage of them. These toxicants are known for causing inhibition on the acetylcholinesterase activity, oxidative stress, interaction with signal transduction pathways, interaction with neurotransmitter receptors, and inhibition of macromolecule synthesis. In fact, chlorpyrifos (CPF), one of the most used ones, has demonstrated its effects on developmental neurotoxicity, which is the main reason for its removal from authorized chemicals in Europe.

Both CYN and CPF could be found in different ecosystems, as aquatic pollutants or in agricultural residues. Thus, the study on the effects of these compounds in combination together the effects of CPF itself was performed, leading to the following publications:

IN VITRO ASSESSMENT OF THE COMBINATION OF CYLINDROSPERMOPSIN AND THE ORGANOPHOSPHATE CHLORPYRIFOS ON THE

HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE (Hinojosa et al., 2020. Ecotoxicology and Environmental Safety, 191, 110222)

TOXIC EFFECTS OF THE CYLINDROSPERMOPSIN AND CHLORPYRIFOS COMBINATION ON THE DIFFERENTIATED SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE (Hinojosa et al., 2021. Toxics. Sent)

Furthermore, as the effects observed were mainly those produced by CPF itself, and this pesticide is known to cause developmental neurotoxicity, a study of the effect of CPF together with CYN was performed after exposure during differentiation to SH-SY5Y cells, by studying viability, effects on the nicotinic acetylcholine receptors, and neurite outgrowth, leading to the following manuscript:

STUDIES OF THE COMBINATION OF THE CYANOTOXIN CYLINDROSPERMOPSIN AND THE PESTICIDE CHLORPYRIFOS ON DEVELOPMENTAL NEUROTOXICITY (Hinojosa et al., 2021. In preparation)

To conclude, taking into account the results derived from the experiments performed in the present Doctoral Thesis, it has been demonstrated the importance of studying the toxics not only isolated but also in combination, as can be found in nature. Furthermore, these results contribute to the toxicological assessment of CYN concerning neurotoxicity.

II.   INTRODUCTION

INTRODUCTION

For years, both climate change and some anthropogenic activities such as urbanization, population growth, deforestation, industrial production or discharge of untreated wastes, etc. are able to cause undesirable effects on the aquatic environment. The main sources of anthropogenic materials to the atmosphere are the combustion of fossil fuels for energy generation, ore smelting, waste disposal such as microplastics, and agriculture. Inputs from agriculture are of special concern, as several pollutants are implied, such as oil and gasoline spills, mobilization of naturally occurring geogenic toxic chemical as heavy metals and metalloids, pesticides, etc. (Champan et al., 1996; Schwarzanbach et al., 2010). Some of the factors causing these changes are particulated, dissolved, or gaseous pollutants reaching the aquatic, terrestrial, and marine ecosystems through different pathways, including atmosphere and soil (Romanelli et al., 2015). In this sense, the uncontrolled and excessive use of fertilizers and pesticides has long-term effects on ground and surface water resources (WHO, 2019). These changes lead to differences in the environmental conditions in surface waters, affecting the growth, physiological performance, and selection of different species of biota (fauna and flora) (Meybeck and Helmer, 1996). Thus, it leads to changes in the species composition of aquatic communities, such as the appearance of different dominant groups, impoverishment of species in the habitat, increased mortality of early life stages, and behavioural or physiological changes (Meybeck and Helmer, 1996). Direct damage to plants and animals nutrition also impacts human health. In this regard, the excess of plant nutrients such as nitrogen or phosphorus, which are involved in the growth of aquatic plant life, could cause the apparition of toxic cyanobacterial blooms (Schwarzanbach et al., 2010; Owa, 2014).

1. Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes and the most abundant group on Earth, capable of producing oxygen and using sunlight as an energy source to convert CO_2 into biomass (Huisman et al., 2018). These organisms are also important in the determination of the path of evolution and ecological changes during the history of the planet, leading to the origin of the eukaryotes in the Cambrian period (Chittora et al., 2020). They can grow in blooms, scums, and biofilms or mats (Fig. 1).

Figure 1. Cyanobloms of *Raphidiopsis raciborskii* and *Microcystis aeruginosa* (from https://freshwaterecology.wordpress.com/2018/03/30/cylindrospermopsis-raciborskii-and-microcystis-aeruginosa-competing-under-different-conditions-of-ph-and-inorganic-carbon/)

This feature is potentiated by hydrological changes in aquatic ecosystems, global warming, and climate change, causing an increase in their incidence (Codd et al., 2017). In the last decades, climate change and anthropogenic activities have led to an increase in the incidence of algal blooms with species that are more toxic and with larger duration and geographical extent, causing major problems for water quality (Heisler et al., 2008; Trevino-Garrison et al., 2015). This overgrowth forms a slime, which causes the reduction of light penetration and oxygenation of water, inducing hypoxia and anoxia for fish, benthic invertebrates, and submerged aquatic vegetation (Huisman et al., 2018). In addition, bad organoleptic properties can be also generated (Khan and Ansari, 2005), which interfere with the use of reservoirs for drinking water or recreational activities in freshwater bodies (Huisman et al., 2018).

The phenomenon by which an excessive growth of aquatic flora such as cyanobacteria occurs due to nutrient enrichment is known as eutrophication (Yang et al., 2008). The main cause for eutrophication is the unloading of high amounts of nutrients such as nitrogen, phosphorus, or carbon into the water bodies, mainly derived from intensified agriculture (Khan and Ansari, 2005). Lately, several studies have pointed out that the increase of CO_2 levels, global warming, and eutrophication increase the frequency, intensity, and duration of cyanobacterial blooms in many aquatic ecosystems across the globe (Huisman et al., 2018). Temperature and pH levels can also increase the rate of cyanobacterial growth.

More than 150 genera and about 2000 different species of cyanobacteria have been identified so far, presenting a wide range of cellular morphologies, physiological capacities, and adaptations (Machado et al., 2017). Many bloom-forming cyanobacteria reach their maximal growth rates above 25°C, faster than eukaryotic phytoplankton, contributing to a longer duration and range expansion of cyanobacterial blooms (Huisman et al., 2018). Consequently, they dwell

in both terrestrial and aquatic ecosystems including extreme habitats (Bownik, 2016; Buratti et al., 2017). Due to their ability to withstand adverse and extreme environmental conditions, together with their capacity for photosynthesis and for fixing gaseous nitrogen into ammonia and amino acids, cyanobacteria are primary colonisers. In fact, cyanobacteria can store essential nutrients such as phosphorus or nitrogen when abundant to be able to grow under nutrient-limiting conditions (Codd et al., 2017).

Their adaptative ability together with their high nutrient content and their small requirement of volumes of water for growth and development, make cyanobacteria an attractive substitute for sustainable food production. In this sense, cyanobacteria can be used as a potential food supplement and provide nutritional, therapeutic and beneficial values. Due to their high content of minerals and vitamins, some species of cyanobacteria, such as *Spirulina, Anabaena,* or *Nostoc*, are consumed as a food supplement in many countries in different pharmaceutical forms like capsules, tablets, and liquids (Chittora et al., 2020). Furthermore, these prokaryotic organisms can have the potential to fix atmosphere nitrogen, which could be used as a biofertilizer for the cultivation of economically important crops. In addition, these organisms are producers of metabolites with pharmaceutical properties, such as antimicrobial, antibacterial, antialgal, antifungal, antiviral, antiprotozoal, antitumoural, cytotoxic, antiinflammatory, antioxidant, antimalarials, antimycotics, herbicides, insecticides, etc. (Demay et al., 2019; Chittora et al., 2020). However, some other compounds like aldehydes, terpenes, ketones, etc., constitute human and animal health hazards (Zanchett and Oliveira-Filho, 2013; Demay et al., 2019). Among these compounds, cyanotoxins are the main concern.
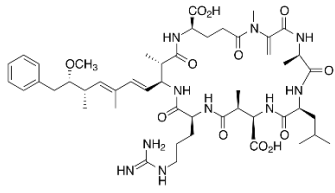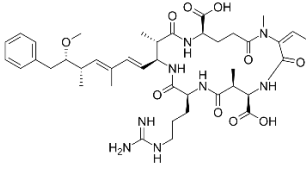
1.1.   Cyanotoxins
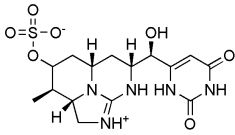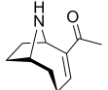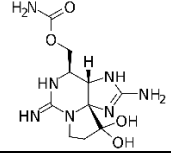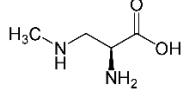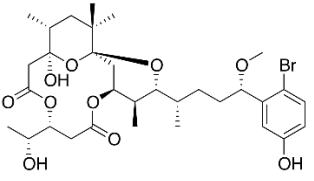
Cyanotoxins are secondary metabolites produced by several species of cyanobacteria able to cause harm in many different organisms (Wood, 2016). These chemicals are of special interest due to their presence in many human and animal intoxication cases (Codd et al., 2017). The production of these toxins has significant adverse effects on aquatic and terrestrial ecosystems, which leads to a reduction of diversity, and also being a potential health hazard to other living organisms, including humans (Wood, 2016; Lee et al., 2017). These toxic metabolites are the main reason for the classification of cyanobacteria as emerging pathogens by the Organization for Economic Cooperation and Development (OECD), in spite of not having the ability to colonize or invade hosts (OECD, 2005; Machado et al., 2017).

Cyanotoxins are usually stored inside the cyanobacteria and their release occurs at the end of the natural lifecycle of the bloom or the cyanobacteria themselves, being the cause of their presence in the water even after the dissipation of the bloom and thus, constituting still a health

risk (Wood, 2016). In addition to the producing species itself, the total production of cyanotoxins concerning their quantity and their profile of cyanotoxin variants relies on a combination of different environmental factors such as temperature, light, and nutrients, among others (Manganelli et al., 2012). Cyanotoxins have been considered in scientific literature as 'secondary metabolites'. However, this label might not be accurate, as secondary metabolites are defined as *"compounds not used by organisms for primary metabolism"*, and there might be some evidence of their current or past potential role in this process (Holland and Kinnear, 2013). The reason for the cyanobacterial production of cyanotoxins has not been elucidated yet. However, they might be a possible evolutionary adaptation for defensive functions against grazing, to reduce competition for resources, cell signaling, iron scavenging, nutrient uptake, homeostasis, or protection against oxidative stress (Holland and Kinnear, 2013; Huisman et al., 2018). In fact, some studies report that the presence of predators has an impact on the level of cyanotoxins-production, varying between toxins even when the same producer is involved (Zanchett and Oliveira-Filho, 2013). The production of these toxicants implicates risks to animal and human health, being able to cause severe poisoning in a short- and long-term. Cyanotoxins are thus important chemical compounds, from an ecotoxicological, toxicological, and environmental points of view (Zanchett and Oliveira-Filho, 2013).

Cyanotoxins vary in their toxicology and they are typically classified according to their main organ target as hepatotoxins, cytotoxins, neurotoxins, or dermatotoxins (Wood, 2016):

Table 1: summary of cyanotoxins based on Codd et al. (2019)

Classification	Cyanotoxin	Structure	Mechanisms of action
Hepatotoxins	Microcystins		Hepatotoxic, tumour promoting, oxidative stress, inhibition of the protein phosphatases PP1 and PP2A
	Nodularin		Similar to MCs
	Cylindrospermopsin		Multiple organ toxicity, neurotoxic, genotoxic, protein synthesis inhibitor
Neurotoxins	Anatoxins		Inhibition on the acetylcholinesterase
	Saxitoxins		Blockage of voltage-gated sodium channels
	β-N-methylamino-L-alanine		Erroneuos insertion into proteins causing misfolding
Dermatotoxins	Aplysiatoxins and lyngbiatoxins		Inflammation and tumour promotion
	Lipopolysaccharides		Inflammatory, promotion of cytokine secretion

1.1.1. Exposure routes

Humans can be exposed to cyanotoxins by several routes (Fig. 2). The main habitat where cyanotoxins can be found is water, so oral exposure through drinking water or contaminated food, or even accidental ingestion during recreational activities, are usually the most accessible route for human exposure to cyanotoxins (Metcalf and Codd, 2012).
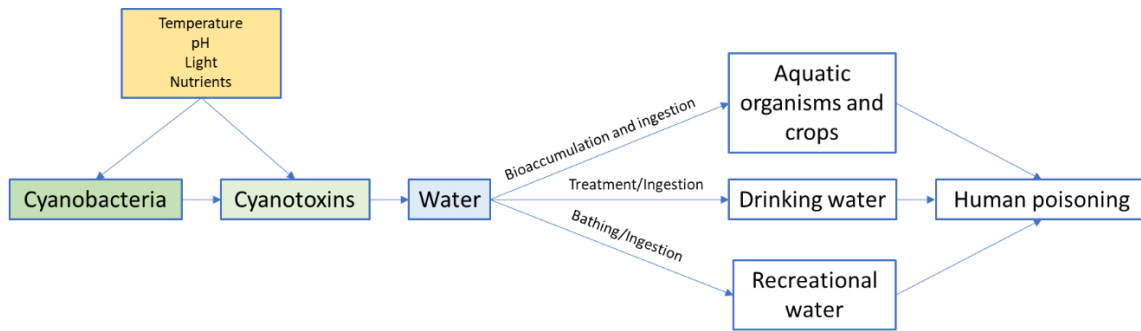
Figure 2. Routes of exposure for cyanotoxins.

Drinking contaminated water is of special concern in arid and developing countries, where inhabitants depend on stored water in reservoirs (Buratti et al., 2017). There are several methods to treat water for cyanotoxin removal, although no single procedure can be recommended as the nature and characteristics of each cyanotoxin are different (Metcalf and Codd, 2012). However, with adequate monitoring and prevention, the risk of exposure can be minimised (Metcalf and Codd, 2012).

Ingestion of contaminated aquatic organisms is another important source of intoxaction (Guzmán-Guillén et al., 2011). Cyanotoxins are of special concern as they may accumulate by their ingestion in edible aquatic organisms such as fish, mussels, or crustaceans, whose safety has not been routinely checked for consumers (Buratti et al., 2017). In this sense, it is known that bivalves are able to concentrate all kinds of toxics present in the water and among them, different types of cyanotoxins (Metcalf and Codd, 2012). Consumption of vegetables irrigated with contaminated water could also be an additional source of exposure, as the soil can also retain the toxins (Lee et al., 2017). Thus, although plants are rarely killed by environmental concentrations of cyanotoxins, their growth and crop yields can be affected, depending their bioaccumulation on the frequency and time of exposure (Prieto et al., 2011; Gutiérrez-Praena et al., 2014; Guzmán-Guillén et al., 2017; Lee et al., 2017). Cyanotoxins can also be present in health-food supplements based on *Spirulina* or *Chrysosporum flos-aquae* grown in artificial ponds or collected from open environments such as natural lakes (Gutiérrez-Praena et al., 2013; Lee et al., 2017).

Dermal and inhalatory routes are also important ways of exposure for cyanotoxins. In this regard, water-based recreational activities such as swimming, paddling, kayaking, or even showering at lakes with presence of cyanobacteria and cyanotoxins have implicated some adverse health outcomes as well (Metcalf and Codd, 2012).

Lastly, the parenteral route has probably been underestimated over the world, as the quality of water used for hemodyalisis is not regulated in most countries despite demonstrating to be the main cause for some cyanotoxin intoxications, among others (Manganelli et al., 2012).

1.1.2. Intoxication reports

Reports of animal poisoning due to cyanotoxins have been documented worldwide for more than a century (Wood, 2016; Díez-Quijada et al., 2019a,b). The first record of a possible animal intoxication by cyanobacterial blooms was carried out by Francis (1878), after the death of livestock and domestic dogs at Lake Alexandrina in South Australia, probably due to nodularin from an extensive bloom of *Nodularia spumigena* had occurred (Metcalf and Codd, 2012). From then, it is known that a wide range of animal species have succumbed to cyanotoxins, including fish, birds, bats, sheep, cattle, horses, pigs, and primates (Wood, 2016). Some reports point out several cases of dog poisoning by anatoxin-a(s) (ATXs) after drinking and bathing in contaminated waters in Scotland, France, Ireland, and USA (Manganelli et al., 2012). Furthermore, there are some cattle death attributed to microcystins (MCs), ATXs, and cylindrospermopsin (CYN) (Mez et al., 1997; Saker et al., 1999; Metcalf and Codd, 2012). Anatoxin-a(s) have been frequently associated with massive mortality of birds (Manganelli et al., 2012).

The documented cases of human poisoning with cyanotoxins have been less frequent but also relevant (Metcalf and Codd, 2012). Most of these cases have been studied retrospectively, with little useful data for the exposure assessment. Nonetheless, epidemiological evidence is highly valuable in elucidating direct connections between cyanotoxins and health effects (Svircev et al., 2017). In this sense, the main cyanotoxins present in short-term human intoxications were MCs in Brazil, England, and Argentina; CYN in Brazil and Australia, and cyanobacterial lipopolysaccharides (LPS) in USA and Brazil, causing mostly gastrointestinal, respiratory, and neuronal symptoms (Metcalf and Codd, 2012). In addition, there are some other cases where the role of cyanotoxins was not confirmed as the main cause of intoxication, but they could be a possible explanation. Even so, increasing evidence indicates that these secondary metabolites also have long-term human health implications, as MCs proved in China or β-N-methylamino-L-alanine (BMAA) in the USA, causing an increase in tumour promotion and neurodegenerative diseases (Metcalf and Codd, 2012).

Among all the cyanotoxins, MCs and CYN are the most frequently detected and responsible for many intoxication cases (Wood, 2016). For this reason, they are the main object of this doctoral thesis.

1.1.3. Microcystins

According to Catherine et al. (2017), about 23 cyanobacterial genera and 47 species are suspected to be MCs-producers. The main genera involved are *Microcystis, Anabaena, Planktothrix/Oscillatoria, Anabaenopsis, Nostoc,* and *Chrysosporum* (De Figueiredo et al., 2004).

The general structure of MCs consists of a cyclo-(D-Ala-X-D-MeAsp-Z-Adda-Arg-D-Glu-Mdha) heptapeptide, where X and Z are variable L-amino acids in positions 2 and 4. D-MeAsp is D-erythro-b-methylaspartic acid, Mdha is methyldehydroalanine and Adda, which is essential for their toxic action, is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Fig. 3) (Chorus and Bartram, 1999; Machado et al., 2017). The overall structure of these toxins is quite hydrophilic due to the carboxylic acids at positions 3 and 6 and the presence of arginine in either 2, 4, or both positions (Catherine et al., 2017). More than 240 MC congeners have been reported to date, with molecular weight varying between 900 and 1100 Da, originating by different amino acid combinations and chemical variations such as methylation/desmethylation of some functional groups (Catherine et al., 2017). The major congeners are MC-LR, MC-RR, and MC-YR, with different combinations of leucine (L), arginine (R), or tyrosine (Y), being MC-LR the most studied due to its ubiquity, prevalence, and toxicity (Campos and Vasconcelos, 2010).
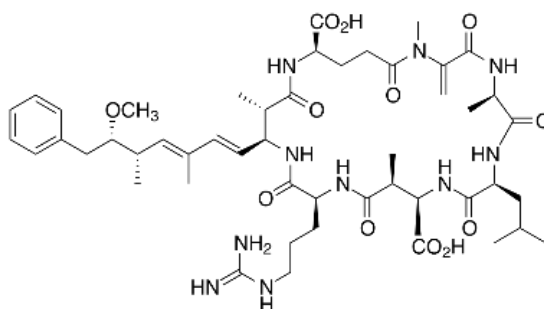
Figure 3. Structure of MC-LR

Microcystins are chemically stable molecules and relatively resistant to degradation, although biodegradation may occur (Metcalf and Codd, 2012). In this line, some species/strains of bacteria reported to degrade MCs in a matter of hours to days are *Sphingomonas, Sphingocinicella, Arthrobacter, Brevibacterium, Rhodococcus,* and *Burkholderia* (Kato et al., 2009; Mazur-Marzec and Plínski 2009). The degradation process starts by attacking the cyclic ring, degradation of the linear peptide to a tetrapeptide, and breakdown of Adda, causing a depletion of its toxicity (Metcalf and Codd, 2012).

The MCs production is highly variable, depending on space and time, making it hard to predict from species composition and cyanobacterial abundance (Huisman et al., 2018). Regarding this, high light intensities and warm temperatures are related to increases in the

transcription of the responsible genes for toxin production (Holland and Kinnear, 2013). For instance, MC-RR production seems to be influenced by high temperatures, while lower ones favour MC-LR synthesis (Rapala et al., 1997; Rapala and Sivonen, 1998; de Figueiredo et al., 2004). The effects of nitrogen and phosphorus concentrations are controversial on MCs content of cyanobacteria, but its production by *Microcystis* strains is influenced by variations in the ratio N:P, with different responses according to the strain (Vézie et al., 2002; de Figueiredo et al., 2004). Microcystins are mostly intracellular, constituting about 70% of the total MCs concentration in raw water (Graham et al., 2010; Buratti et al., 2017). However, high extracellular concentrations can be found because of blooms lysis (Rohrlack and Hyenstrand, 2007; Díez-Quijada et al., 2019b). These concentrations vary considerably both from one bloom to another and during the course of a single bloom (Buratti et al., 2017).

In addition, genotype diversity between strains seems to be the main factor affecting toxicity levels in blooms of the same species (Rohrlack et al., 2001; Kurmayer et al., 2002; de Figueiredo et al., 2004). In this respect, the genotypes can differ in growth strategy, interaction with zooplankton, plasmids, MCs synthetase genes cluster, and MCs content, leading to different MCs variants with different toxic properties (Hesse and Kohl, 2001; Mikalsen et al., 2003; de Figueiredo et al., 2004).

Concerning the purpose of MCs synthesis, there exist different explanations (de Figueiredo et al., 2004). They could act as a chemical defense against grazing (Kurmayer and Jüttner, 1999; Henning et al., 2001), have an allelopathic effect over algal competitors (Kearns and Hunter, 2001), as a nitrogen reserve or as a regulator of endogenous protein phosphatases (de Figueiredo et al., 2004). However, this remains still unclear (Humbert and Fastner, 2017).

To avoid human toxicity, the World Health Organization (WHO) released a provisional drinking water guideline of 1 µg/L for MC-LR (WHO, 2020a). This value has been adopted as a guideline in many countries. However, some countries such as Canada and Australia proposed 1.5 mg/L and 1.3-10 mg/L, respectively (USEPA, 2001). Furthermore, Canada also proposed for short-term exposure the value of 10 mg/L (Fitzgerald, 2001; de Figueiredo et al., 2004). Also, WHO set the TDI value of 0.04 μg/kg bw/ day. Based on updated data, the Environmental Protection Agency (EPA, 2006) in USA proposed acute and chronic TDI guidelines (0.006 and 0.003 MC-LR µg/ kg bw/day, respectively).

1.1.3.1. Geographical distribution

The distribution and concentration in waterbodies of MCs depend on the biomass of the producer cyanobacteria, the amount and congeners in the cells, the share of toxigenic genotypes

in a population, and the release of extracellular MCs into the water (Humbert and Fastner, 2017). Different MC congeners are frequently found at the same time in the same bloom (Catherine et al., 2017). Blooms of MC-producing cyanobacteria have been reported in 80 countries, being more relevant in Europe and North America (Fig. 4) (Buratti et al., 2017; Catherine et al., 2017).
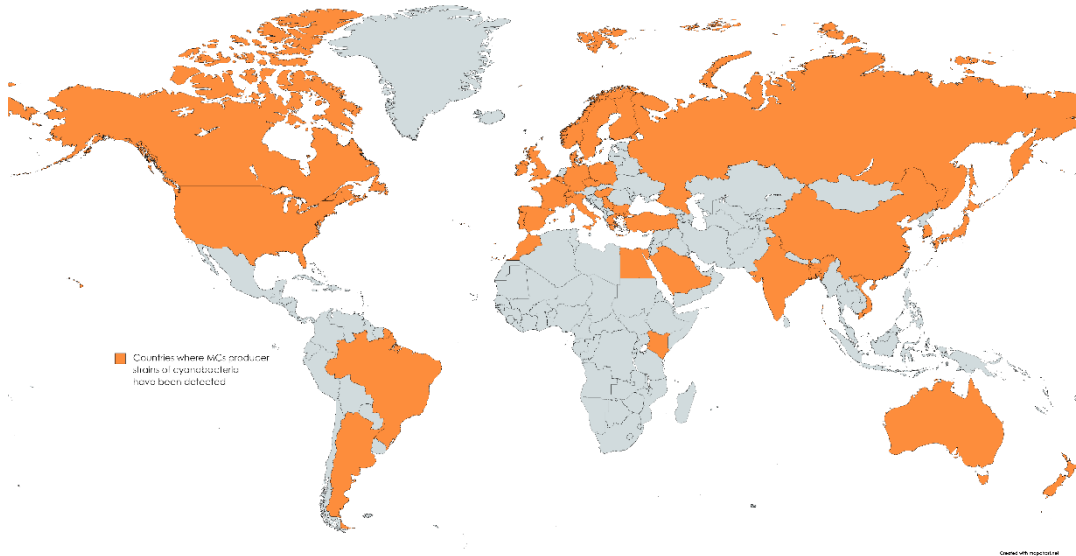
Figure 4. Blooms of MCs producers - cyanobacterial strains.

In general, MCs are the most common cyanotoxins, with levels up to 126.42 µg/L in Asia, 11400 µg/L in Europe, and 10000 µg/L in the USA (Buratti et al., 2017).

1.1.3.2. Bioaccumulation of MCs

In general, bioaccumulation patterns have been demonstrated to depend on the species, probably due to interspecific differences in selective ingestion and depuration rates (Kim et al., 2017; Díez-Quijada et al., 2019a). Among all the organisms able to bioaccumulate MCs, bivalves are very important due to their filtering capacity, providing a relevant human health threat since they are consumed on a regular basis in many countries (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Gutiérrez-Praena et al., 2013). Thus, the edible mussel *Mytilus galloprovincialis* has been shown to accumulate higher quantities of MCs than the tolerable daily intake (TDI) established by WHO, which is of 0.04 µg/kg/day, with the consequent risks to humans (WHO, 1998; 2020a). In addition, crustaceans feed on phytoplankton, including cyanobacteria, so their metabolites can accumulate in their tissues. There are several studies of bioaccumulation in different species of crustaceans reporting higher values of MCs than those recommended as TDI by WHO (WHO, 1998; Chen and Xie, 2005; García et al., 2010). Normally, bivalves and crustaceans would be safer for humans if the target organs of MCs were removed before consuming them, but these

organisms are usually fully eaten, providing risky levels of MCs for humans (Gutiérrez-Praena et al., 2013).

Additionally, some of the most important aquatic organisms consumed worldwide are fish. Fish can be exposed to cyanotoxins by directly feeding on phytoplankton or food web, or by uptaking dissolved toxins via epithelium (Ibelings and Chorus, 2007; Gutiérrez-Praena et al., 2013). Fish are able to bioaccumulate toxins mainly in liver and muscle (Magalhães et al., 2001; Mohamed et al., 2003; Gutiérrez-Praena et al., 2013). In this respect, different studies on tilapia (*Oreochromis niloticus)* and carps (*Cyprinus carpio*) exposed to MCs-producing blooms in different reservoirs found that the TDI value was exceeded in most of the samples analyzed. The higher MCs concentrations were found in guts > liver > kidney > muscle, changing the concentrations according to the different depuration abilities of the fish (Mohamed et al., 2003; Chen 2006, 2007; Deblois et al., 2008; Guzmán-Guillén et al., 2017). Although is not the main organ containing higher concentrations of MCs, muscle has demonstrated to bioaccumulate higher concentrations than the TDI, constituting a potential human health risk (Gutiérrez-Praena et al., 2013).

In addition, MCs bioaccumulation by aquatic animals is a multifactorial process depending on their feeding guild, physiology, length, time of exposure, accumulation, depuration, and metabolic rates of the fish species (Jia et al., 2014; Díez-Quijada et al., 2019a). Furthermore, it has also been demonstrated that different cooking procedures (microwave oven, boiling, steaming etc.) can affect the level of different MC-congeners reaching humans (Zhang et al., 2010; Guzmán-Guillén et al., 2011; Buratti et al., 2017).

Although aquatic ecosystems are an important source of cyanotoxins-bioaccumulation, cyanobacterial blooms can also appear in water reservoirs for plant irrigation. In this sense, plants can also be exposed to MCs and accumulate them (Gutiérrez-Praena et al., 2013). Several studies performed in different plant species such as mustard, broccoli, clover, rape, parsley, rice, apple, spinach, or lettuce among others, demonstrated their capacity for MCs-bioaccumulation and the effects of these cyanotoxins in their physiology (Kós et al., 1995; Crush et al., 2008; Mohamed and Al Shehri, 2009; Chen et al., 2010; 2012; Prieto et al., 2011; Gutiérrez-Praena et al., 2013; Freitas et al., 2015; Buratti et al., 2017). Crush et al. (2008) also concluded that plants with big leaves retain more contaminated water than the ones with small leaves. However, edible plants accumulate MCs not only on the leaves but also in the roots and sprouts (Díez-Quijada et al., 2019a). In general, the results obtained predict the exposure of humans to higher concentrations than the TDI for MCs (Gutiérrez-Praena et al., 2013, WHO, 2020a).

1.1.3.3. Intoxication reports

The routes of exposure are common for most cyanotoxins. In addition, it is important to take into account the time of exposure, as in the case of MCs can lead to acute or chronic effects. Microcystins seem to have been involved in several chronic human exposure cases around the globe. In this regard, there are many cases of animal intoxications associated with cyanobacterial blooms and scums through dermal contact. Over the past 60 years, there are many records of the death of fish, waterbirds, turtles, terrapins, otters, sheep, cattle, roe deers, rhinos, zebras, wildebeests, and dogs related to several MCs-congeners (Svircěv et al., 2017; Buratti et al., 2017).

In general, acute intoxications due to MCs have been reported in many countries in the last century, such as Canada, Sweden, and USA in the case of animals, and USA, UK, Brazil, and Argentina in the case of humans. In general, MCs intoxication led to gastrointestinal, respiratory, and nervous symptoms. Among them, fever, malaise, weakness, nausea, abdominal cramps, vomiting, painful diarrhoea, hepatomegaly, liver failure, headache, muscle and joints pain, facial rashes, blistering around the mouth, dry cough, sore throat, dyspnea and respiratory distress, lethargy, myalgia, difficulty walking, visual disturbances, mild deafness, confusion, episodes of hallucinations and convulsions were the most common ones (Tisdale 1931; Dillenberg and Dehnel 1960; Turner et al., 1990; da Teixeira et al., 1993; Pouria et al., 1998; Codd et al., 1999; Annadotter et al., 2001; Carmichael et al. 2001; Azevedo et al. 2002; Soares et al., 2006; Dietrich et al. 2007; Gianuzzi et al., 2011; Bautista et al., 2015; Trevino-Garrison et al. 2015; Buratti et al., 2017; Svircěv et al., 2017).

Regarding chronic exposure, some studies have found a correlation between MCs-producers blooms and different types of carcinoma in USA and China after exposure mainly through oral route, as Svircěv et al., 2017 indicate in their review. Due to these large exposure periods, MCs are suspected to be one of the main factors associated with the high incidence of hepatocellular carcinoma, liver, stomach, and colorectal cancer, and renal function impairment since the 1980s, according to different studies performed in thousands of inhabitants (Svircěv et al., 2017). In this sense, the same authors reported a possible indirect link between MCs-producer cyanobacterial blooms and the incidence of primary liver cancer or non-Hodgkin's lymphoma through drinking water in Serbia. This carcinogenicity is probably due to a synergistic effect with some other factors also present in the environment. Moreover, in Portugal, from 2000 to 2008, where cyanobacterial blooms of *M. aeruginosa*, *Aphanizomenon* spp., and *Oscillatoria* were detected (Bellem et al. 2013), an increase of the biomarkers for liver damage and a rise of different types of cancer were also reported (Bellem 2014; Svircěv et al., 2017). Furthermore, in Africa, Gunnarsson and Sanseovic (2001) reported a possible connection between cyanobacterial species and MCs in drinking water and diarrhoea and elevation of liver enzyme activities in serum

(Svircěv et al., 2017). All of this lead to its classification as a possible carcinogenic to humans (class 2B) by the International Agency for Research on Cancer (IARC) (IARC, 2010; Machado et al., 2017).

1.1.3.4. Toxicity of MCs

1.1.3.4.1.   Toxicokynetics

Due to their high molecular weight and structure, MCs cannot readily diffuse through plasma membrane (Campos and Vasconcelos, 2010). However, the specific struture and physico-chemical properties of each MC-congener influence their kinetic process. Thus, MC-LR is highly hydrophilic and cannot penetrate cell membranes by passive transport; its kinetic parameters may be significantly different from those of more lipophilic MC congeners (Testai et al., 2016; Díez-Quijada et al., 2019b). When MC-LR enter the organism through the oral route, they are transported across the ileum into the bloodstream through the bile-acid transporter of hepatocytes and cells of the small intestine or partially in the stomach (Falconer and Yeung 1992; de Figueiredo et al., 2004; Svircěv et al., 2017). Then, most of the MC-LR content is reabsorbed into the portal bloodstream to the liver, where the organic anion transporting polypeptide superfamily (OATPs) mediates their uptake into the hepatocytes (via Oatp1b2, OATP1B1, and OATP1B3) and across the blood-brain-barrier (OATP1A) (Hagenbuch et al. 2002; Fischer et al. 2005; Komatsu et al. 2007; Lu et al. 2008; Svircěv et al., 2017). Over 300 OATPs/Oatps have been reported in different species (Hu et al., 2016), but only four out of eleven OATPs reported in humans (OATP1A2, -1B1, -1B3, and -2B1) have been shown to be involved in the uptake of MCs (Fischer et al., 2010; Díez-Quijada et al., 2019b). Although the primary organ affected by MC-LR is the liver, there are effects at many other levels. This the case of the gastrointestinal tract, reproductive organs, kidneys, lungs, heart, and skin of rodents (Slatkin et al., 1983; Falconer and Buckley, 1989; Fujiki et al., 1989; Falconer, 1991; Nobre et al., 1999; Humpage et al., 2000; Milutinovic et al., 2002; 2006; Botha et al., 2004; Ding et al., 2006; Soares et al., 2007; Li et al., 2009a; Svircěv et al., 2017). The absorption process is not equal for all the MC-congeners, they can have different affinity to different OATPs across the membrane, which could explain their differences in toxicity (Monks et al., 2007; Feurstein et al., 2010; Fischer et al., 2010; Bulc Rozman et al., 2017; Díez-Quijada et al., 2019b). In general, the OATP is a multispecific transport system present in different cell types and organs (Campos and Vasconcelos, 2010). Thus, the systemic distribution of MCs is dependent on the degree of blood perfusion and types and expression level of OATP carriers (Campos and Vasconcelos 2010). In this sense, OATP1B1 and 1B3 are human liver-specific while OATP1A2 is highly expressed in endothelial cells of the

blood-brain-barrier (BBB), epithelial cells of the blood-cerebrospinal-fluid-barrier and in the cell membrane of human neurons (Feurstein et al., 2009; Campos and Vasconcelos, 2010).

Metabolism and excretion pathways of MC-LR include glutathione (GSH) conjugation, being the kidneys the main organ for their excretion and, thus, an important target for MC-LR toxicity (Buratti et al., 2017; Díez-Quijada et al., 2019b). In fact, glutathione-MCs, Cys-Gly-MCs, and Cys-MCs have been identified after incubation with glutathione-S-transferase (GST) from a wide range of plants and animals *in vitro* as products of MCs-detoxification (Pflugmacher et al., 1998; Wiegand and Pflugmacher 2005; Campos and Vasconcelos, 2010). However, although the GSH conjugate formation reaction is commonly catalyzed by GST, it can also occur spontaneously (Kondo et al., 1992; Roegner et al., 2014; Testai et al., 2016; Díez-Quijada et al., 2019b).

1.1.3.4.2.   *Mechanisms of action*

Microcystin-LR has demonstrated to cause toxicity through several mechanisms of actions (Fig. 5):
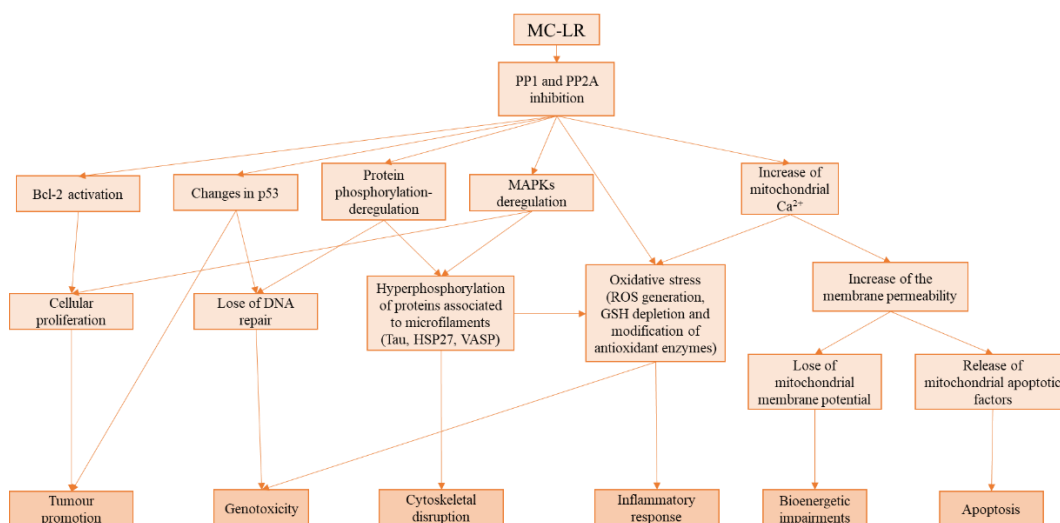
Figure 5. Mecanisms MC-LR toxicity

- The main one consists of the **irreversible inhibition of Serine/Threonine protein phosphatases 1 and 2A** (PP1 and PP2A) by covalent binding (MacKintosh et al., 1990; Dawson, 1998; Machado et al., 2017). First, a non-covalent binding between the ADDA moiety of MCs and the active center of PP1 and PP2A occurs, which seems to be the step responsible for the main inhibitory effect of the toxin (Machado et al., 2017). Then, there is a covalent binding between the Mdha residue of the toxin and the cys-273 of the catalytic subunit of PP1 or the cys-266 of the catalytic subunit of PP2A

(Craig et al., 1996; MacKintosh et al., 1990, 1995). In general, the protein phosphorylation and dephosphorylation is a dynamic process catalyzed by phosphatases and kinases for the regulation of protein activity in cells (Campos and Vasconcelos, 2010). Thus, PP2A inhibition can cause an impact on cellular homeostasis (Campos and Vasconcelos, 2010).

- Mediation in the **expression of mitogen-activated protein kinases (MAPKs)**, which regulate the expression of proto-oncogenes involved in the growth and differentiation (Gehringer, 2003; Campos and Vasconcelos, 2010).

  o The MAPKs activation causes **hyperphosphorylation** of different types of microfilament-associated proteins, being this the case of Tau, HSP27, and vasodilator-stimulated phosphoprotein (VASP) (Zeng et al., 2015; Sun et al., 2015). This hyperphosphorylation has an impact on their ability to bind and stabilize cellular cytoskeleton (Buratti et al., 2017). In this regard, high concentrations of MC-LR have been reported to cause aggregation of cytokeratin intermediate filaments and actin microfilaments around the nucleus (Falconer and Yeung 1992; Ghosh et al. 1995; Clark et al. 2007), which would modify the cytoskeleton (Buratti et al., 2017).

  o MAPKs-deregulation also stimulates the **synthesis and phosphorylation of transcription factors**, including FOS and JUN, that leads to the production of growth factors, receptors for growth factors, and proteins controlling the entry of cells into the cell cycle (Kumar et al. 2005; Svircěv et al., 2017). In general, the alteration of cell proliferation, division, signal transduction, and gene expression caused by the hyperphosphorylation of transcription factors *c-Myc* and *c-Jun*, has been reported (Liu et al., 2016; Buratti et al., 2017). In this sense, MCs have been demonstrated to induce the overexpression of those proto-oncogenes (Li et al., 2009a; Wang et al., 2013; Buratti et al., 2017). This tumor-promoting activity of MC-LR after PP2A inhibition has been observed after the activation of some factors such as the tumor necrosis factor alpha (TNF-α), an endogenous tumor promoter and a central mediator of tumor promotion (Fujiki and Suganuma, 2011; Zhang et al., 2013; Buratti et al., 2017).

- **Loss of activity of DNA-dependent protein kinase (DNA-PK)**, due to deregulation of phosphorylation mechanisms, which would decrease the cellular capacity to repair DNA double-strand break, which would favour their genotoxicity and tumor promotion features (Douglas et al. 2001; Kleppe et al. 2015; Buratti et al., 2017).

- **Oxidative stress**, which could be a consequence of the activation of calpin and Ca^{2+}/calmudin-dependent kinase 2 (CaMKII), being activated through the increase of

intracellular Ca^{2+} (Ding et al., 2000; 2003). CAMKII activation occurs by inhibiting its dephosphorylation, a process that may be led by PP1 and PP2A. In addition, CaMKII activation can also regulate reactive oxygen species (ROS) formation and phosphorylation of proteins (Krakstad et al., 2006; Campos and Vasconcelos, 2010). In this regard, the studies reporting the MCs capacity to increase the production of ROS are wide (Pflugmacher, 2004; Pflugmacher et al., 2006, 2007a,b; Pichardo and Pflugmacher, 2011; Žegura et al., 2011; Zhou et al., 2015), modifying intracellular antioxidant enzymes (Zanchett and Oliveira-Filho, 2013). Furthermore, some other possible explanations for the production of oxidative stress by MC-LR could be that GSH is used as a driving force for the exchange with MCs in its incorporation into the cell via OATPs (Amado and Monserrat, 2010). This decrease of the GSH levels could also cause, therefore, an increased ROS generation. Cellular stress can **induce the nuclear phosphoprotein p53**, which is a transcriptional trans-activator in DNA repair, apoptosis, and tumor suppression regulated by PP2A as well (Fu et al., 2005; Li et al., 2007). Its main function is the regulation of the expression of anti- and pro-apoptotic genes including Bcl-2 and Bax (Campos and Vasconcelos, 2010). Thus, the consequence of p53 induction would be the activation of Bcl-2 protein and the protein kinase c (PKC) (Svircěv et al., 2017). This activation ends in the increase of cell survival and proliferation, which may lead to tumour promotion (Deng et al., 1998; Svircěv et al., 2017). The DNA damage produced by MC-LR and reported in many *in vitro* and *in vivo* studies, seems to be associated with ROS formation, mutagenic oxidative DNA lesions, interference with DNA repair, and clastogenic activity. This would suggest the oxidative stress induction as a consequence of tha damage instead of a direct interaction between MCs and DNA (Funari and Testai 2008; Amado and Montserrat, 2010; Žegura et al. 2011). In addition, the main effect observed after exposure to low doses of MC-LR is cell proliferation, sublethal exposure is associated with ROS formation that leads to apoptosis, and very high doses are able to cause hepatocyte necrosis (Svircěv et al., 2010; 2017).

1.1.4. Cylindrospermopsin

Cylindrospermopsin is also an important cyanotoxin due to its increased incidence in the last decades. However, the available studies concerning its toxicological profile and bioaccumulation are much less large than those available for MCs.

Cylindrospermopsin consists of a tricyclic guanidine moiety combined with a hydroxymethyl uracil (Ohtani et al., 1992) (Fig. 6). This alkaloid is a sulfated guanidinium zwitterion with a relatively low molecular weight (415 Da), making it highly water-soluble and

stable in varying heat, light, and pH conditions (Chiswell et al., 1999; Kinnear, 2010). Its biological role is thought to be a direct competitive advantage by grazing defense or allelopathy against other cyanobacterial populations for resource competition, light, or nutrients (Holland and Kinnear, 2013; Kaplan et al., 2012). According to Bar-Yosef et al. (2010), CYN leads to the secretion of alkaline phosphatase by other phytoplankton, increasing the amount of inorganic phosphate available to CYN-producer cyanobacteria, giving these species the advantage to outcompete other species with limited inorganic phosphate (Kokociński et al., 2019). Furthermore, four structural variants of CYN, 7-epi-CYN, deoxy-CYN, 7-deoxy-desulfo-CYN, and 7-deoxy-desulfo-12-acetylCYN have been described (Wimmer et al., 2014). To date, it is still not known if they are precursors, variants or degradation products, although they have demonstrated to exert less toxicity (Li et al., 2001; Norris et al., 1999; Sukenik et al., 2001).
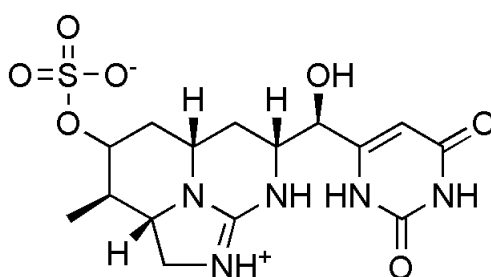
Figure 6. CYN chemical structure.

1.1.4.1. Geographical distribution

Due to the slow degradation of CYN in the water, its possible membrane permeability, and its water-solubility, this cyanotoxin has been found mainly as an extracellular fraction, being able to be released into water bodies up to 100% of total CYN (Sivonen et al., 1999; Yang et al., 2021). Concerning the environmental CYN-concentrations, the highest value reported is 1050 µg/L from a water supply in central Queensland, Australia (Yang et al., 2021), being able to affect both humans and the environment (Pichardo et al., 2017a). However, CYN levels up to 97 µg/L have been detected in finished drinking-water, although the investigation concerning these levels is quite limited so far (WHO, 2020). For this reason, the values proposed by WHO (2020) of 0.7 µg/L in lifetime drinking-water, and 3 µg/L in short-term drinking water.

Cylindrospermopsin is an emerging threat worldwide due to the progressive distribution of *Cylindrospermopsis raciborskii,* recently renamed as *Raphidiopsis raciborskii*, its main producer (Kinnear, 2010; Poniedzialek et al., 2012; Machado et al., 2017; Aguilera et al., 2018). Taking into account that *R. raciborskii* barely changes the colour of the water, can be difficult to

recognize their blooms for the naked eye (Yang et al., 2021). Nonetheless, this cyanotoxin has been also detected in different species, causing an increase in its presence around the globe (Fig. 7). In general, CYN has demonstrated to be present in higher concentrations in freshwaters influenced by human activities (WHO, 2020).
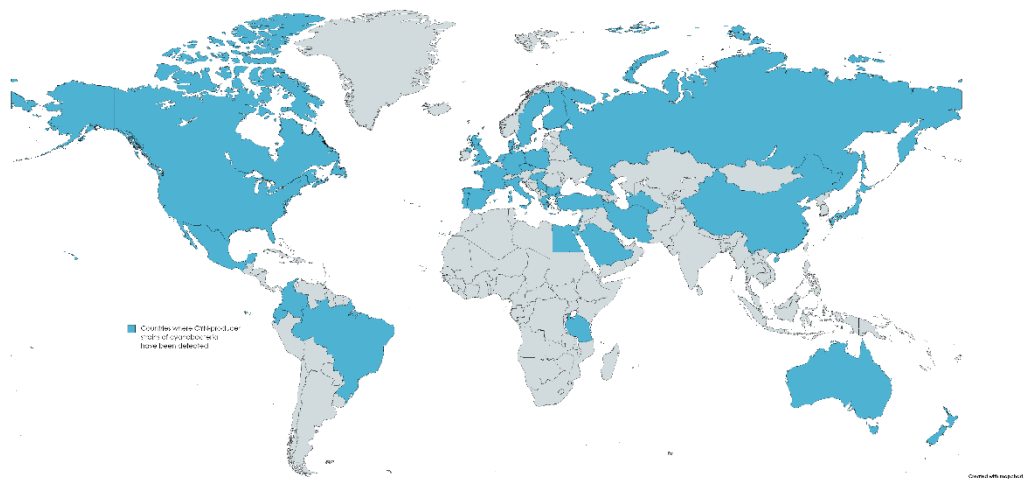
Figure 7. Blooms of CYN producers- cyanobacterial strains

Different studies have been performed in order to elucidate the effects of environmental factors on CYN production, such as temperature, nitrogen and phosphate sources, and light intensity, which have been demonstrated to vary among the different CYN-producers (Kokociński et al., 2019). In general, these producers-organisms can be found in lakes, reservoirs, rivers, ponds, and dams (Kokociński et al., 2019). In regard, their adaptability together with climate change and increasing eutrophication is allowing these species to expand into habitats that were not suitable before, making it likely to lead to an ever-increasing incidence of CYN in the future (Scarlett et al., 2020). Furthermore, the CYN-congeners deoxy-CYN and 7-epi-CYN have been detected in cyanobacterial blooms of *R. raciborskii*, *R. curvata*, *R. mediterranea*, *A. ovalisporum*, and *Oscillatoria* spp. (Mazmouz et al., 2010; Mc Gregor et al., 2011; Li et al., 2001; Norris et al., 1999; Banker et al., 2000; Kokociński et al., 2019).

1.1.4.2. Bioaccumulation of CYN

The bioaccumulation studies available are scarce and dependent on the species and the CYN conditions (purified, in a cell extract, in a producer species, etc.), although the results are worth mentioning. In this sense, CYN has demonstrated its ability to be bioaccumulated by a range of organisms from different parts of the food chain, including mollusks, fish, toads, and plants (Kinnear, 2010). In mollusks, Saker and Eaglesham (1999) exposed *Anodonta cygnea* to

14-90 µg/L CYN for 16 days, obtaining bioaccumulation in haemolymph, viscera, mantle, foot, and gonads (Kinnear, 2010). Furthermore, after 14 days of depuration, almost 50% of the toxin remained in the tissues (Saker and Eaglesham, 1999; Kinnear, 2010). Likewise, Anderson et al. (2003) reported CYN accumulation levels of 130-560 µg / kg in *Alathyria pertexta* after being exposed to less than 0.8 µg/L CYN (Kinnear, 2010). Concerning the study performed by Berry and Lind (2010) in Tegogolo snails (*Pomecea patula catemacensis*), their field CYN exposure gave a BCF value of 157, while the environmental concentrations measured were of 20 ng/L CYN, demonstrating their bioaccumulation even at low CYN concentrations (Berry and Lind, 2010; Kinnear, 2010). In addition, White et al. (2006) studied the CYN-bioaccumulation in *Melanoides tuberculata* exposed over seven and fourteen days (Kinnear, 2010). They also found much higher bioaccumulation when exposed to live cultures of *R. raciborskii* than to cell extracts containing the aqueous toxin, in agreement with the results obtained by Seifert (2007) in *Lemna puntata* (White et al., 2007; Kinnear, 2010). Moreover, White et al. (2007) reported bigger concentrations of deoxy-CYN than of CYN (Kinnear, 2010). This is in agreement with the results obtained by Seifert (2007) who observed a much bigger bioaccumulation value for muscle and viscera in *Corbiculina australis* mussels for deoxy-CYN than for CYN when exposed to a known CYN-producing bloom (Kinnear, 2010). The studies concerning CYN-bioaccumulation in crustaceans also point out its ability to stay in the organism. In this sense, Saker and Eaglesham (1999) detected CYN values of 900 and 4300 µg/kg freeze-dried tissue from muscle and hepatopancreas, respectively, in crayfish *Cherax quadricarinatus* from an aquaculture pond containing 589 µg/L CYN (Kinnear, 2010). Seifert (2007) confirmed these results in *Cherax* crayfish (Kinnear, 2010). However, the study performed in *Daphnia magna* exposed to *C. raciborskii* lead to a non-significant CYN-bioaccumulation after 24 and 48 hours (Nogueira et al., 2004). In the bioaccumulation studies using fish, the results are also species-dependent. The first authors reporting CYN bioaccumulation were Saker and Eaglesham (1999), in rainbow fish *Melanotaenia eachamensis*, with values of 1200 µg/kg freeze-dried tissue. Furthermore, Seifert (2007) reported the bioaccumulation of both CYN and deoxy-CYN in eel-tailed catfish (*Tandanus tandanus*), but not in Australian bass (*Macquaria novemaculeata*), golden perch (*M. ambigua*), or silver perch (*Bidyanus bidyanus*) (Kinnear, 2010). In this line, Berry et al. (2012) detected CYN in muscle tissues from different fish species such as *Bramocharax caballeroi, Cichlasoma uropthalmus, C. helleri, Dorosoma mexicana, Heterandria jonesii, Oreochromis aureus, Rhamidia* sp*., Vieja* sp*.* and *V. finestrata* (Berry et al., 2012; Scarlett et al., 2020). In addition, Guzmán-Guillén et al. (2015) reported bioaccumulation of CYN after 14 days of exposure in the brain of tilapia, reducing the levels after 3 and 7 days of depuration (Guzmán-Guillén et al., 2015). This is in agreement with the detection of CYN in muscle, liver and brain of *Hoplias malabaricus* exposed for 7 and 14 days to both purified CYN and CYN-producing cyanobacterial extract (da Silva et al., 2018). Moreover, there is only one study concerning the CYN-bioaccumulation in

tadpoles of the cane toad *Bufo marinus*. In this experimental model, White et al. (2007) reported BCF of 19.27 after exposure to live *C. raciborskii* cultures containing CYN, although no bioaccumulation was detected after their exposure to whole-cell extracts (White et al., 2007; Kinnear, 2010). Regarding the studies concerning CYN-bioaccumulation by plants, White et al. (2005) and Kinnear et al. (2007) reported little concentrations of CYN after exposure to *Hydrilla verticillata* and *Lemna punctata*, respectively (Kinnear, 2010). In this aspect, Seifert (2007) also reported in *Lemna punctata* a low bioconcentration factor (BCF). However, when cell extracts were used instead of the purified toxin, the BCF values were up to 86.67 (Kinnear, 2010). Furthermore, the BCF decreased with increasing CYN exposure concentrations in arugula (*Eruca sativa* Mill.) and lettuce (*Lactuca sativa* L.) after 3, 5, and 10 mg/L (Cordeiro-Araújo et al., 2017).

For all of this, a provisional TDI of 0.03 µ/kg bw has been established for CYN (WHO, 2020).

1.1.4.3. Intoxication reports

The most famous case of human intoxication due to CYN was through contaminated drinking water, causing an outbreak of hepatic enteritis on Palm Island, Queensland, Australia, known as "Palm Island Mystery Disease" in 1979. In this incident, over 139 children and 10 adults showed various symptoms of gastroenteritis, such as anorexia, vomiting, headache, hepatomegaly, initial constipation followed by bloody diarrhea and dehydration (Byth, 1980; Hawkins et al., 1985; Griffiths and Saker, 2003; Codd et al., 2016). This case occurred after a few days of the treatment with copper sulfate of the Solomon Dam reservoir to control an algal bloom containing *R. raciborskii*, from where all the patients reported the intake of drinking water. This water treatment caused cellular lysis, and thus, the release of CYN (Metcalf and Codd, 2012; Zanchett and Oliveira-Filho, 2013). Furthermore, CYN was also postulated to be implied in the Caruaru syndrome. However, as no CYN quantification was reported, its contribution to the toxic effects observed cannot be evaluated (Carmichael et al., 2001; Buratti et al., 2017). In addition, there are cases of animal intoxication by contaminated water intake, causing the death of fish, birds, and cattle (Yang et al., 2021).

1.1.4.4. Toxicity of CYN

1.1.4.4.1.   *Toxicokynetics*

Due to its hydrophilic properties, CYN is unlikely to cross the lipidic bilayer-membrane cell, requiring the mediation of the bile acid transport system (Runnegar et al., 2002; Froscio et

al., 2009; Pichardo et al., 2017b). However, passive diffusion through biological membranes has also been demonstrated because of its small molecular weight (Chong et al., 2002; Buratti et al., 2017). Cylindrospermopsin uptake varies among different types of cells, as it seems to be fast in primary mouse hepatocytes (Froscio et al., 2003) and slow in kidney or colon cells (Froscio et al., 2009a,b; Fernandez et al., 2014). In this sense, the main pathway involved in CYN uptake in intestinal cells seems to be the paracellular route, with a minor carrier-mediated transcellular transport H^+ and GSH-dependent (Pichardo et al., 2017a,b). However, this uptake has not been reported so far in other cell lines and in Caco-2 cells is very limited (Pichardo et al., 2017a,b).

In general, the differences in the toxicity exerted by CYN in different experimental models could be linked to their metabolic capacity (Pichardo et al., 2017a). Regarding this, although no studies have demonstrated direct CYN biotransformation, the cytochrome P450 (CYP450) enzymatic system seems to be involved in the bioactivation of CYN, as some reports have demonstrated with both inducers and inhibitors of CYP450 on CYN toxicity (Norris et al., 2002; Froscio et al., 2003; Humpage et al., 2005; Bazin et al., 2010b). Accordingly, some authors have reported that S9 fraction-inducing metabolism led to an increase in the cytotoxic and genotoxic effects of CYN (Runnegar et al., 1994; Lankoff et al., 2008; Froscio et al., 2003; Puerto et al., 2018; Pichardo et al., 2017a). Furthermore, it has been demonstrated the ability of CYN to upregulate genes coding for phase I enzymes (CYP1A1, CYP1B1, ALDH1A2, and CES2) and phase II enzymes (UGT1A6, UGT1A1, NAT1, and GSTM3) in HepG2 cells (Štraser et al., 2013). However, Ließel et al. (2015) reported no increase in CYN toxicity after pre-inducing the expression of CYPs. In addition, Kittler et al. (2016) reported no phase I biotransformation after exposure of HepaRG cells, a metabolically competent human cell line, to CYN after 24 hours. Nonetheless, when exposed to the CYP3A4 inhibitor ketoconazole, the cytotoxicity was significantly decreased (Kittler et al., 2016). Besides, a substantial GSH depletion was observed after oral CYN administration to rats, meaning a possible CYN conjugation with GSH or a GSH synthesis-inhibition by CYN (Runnegar et al., 1995; Buratti et al., 2017).

Concerning its excretion, both urinary and fecal pathways have demonstrated to be quite rapid, requiring 0-12 hours for a complete excretion, not being almost observable after 24 hours (Norris et al., 2001). However, the studies regarding its elimination are still limited (WHO, 2020).

1.1.4.4.2. *Mechanisms of action*

The mechanisms of action for CYN are not well elucidated yet (WHO, 2020). However, it is known that they depend on different factors such as the magnitude and frequency of dose,

exposure duration, and the characteristics of the exposed organism, such as life stage, age or sex (WHO, 2020). In this regard, CYN seems to act through different mechanisms (Fig. 8):
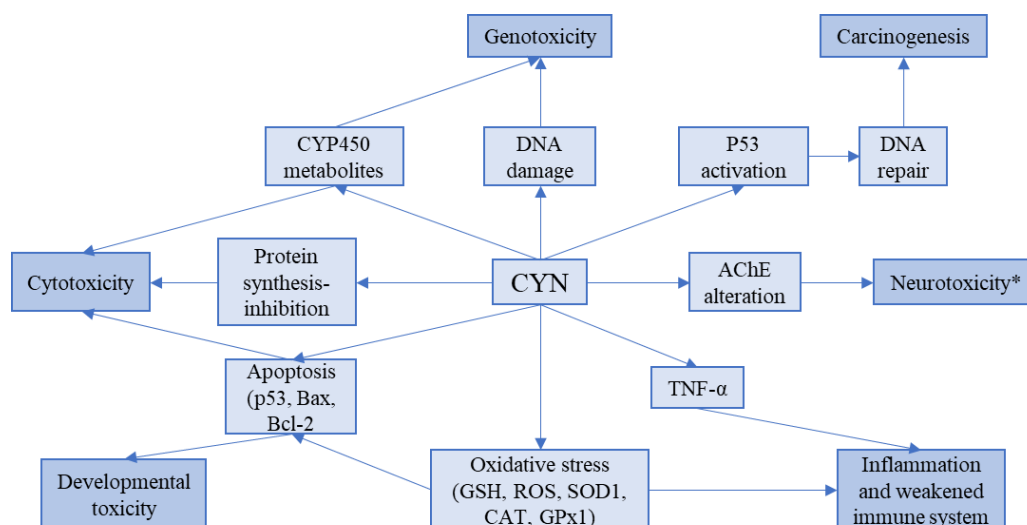

Figure 8. Mecanisms CYN toxicity (based on Yang et al., 2021)

- Complete and irreversible concentration-dependently **protein synthesis-inhibition**, both *in vitro* and *in vivo*, which leads to hepatic and renal (cyto)toxicity (Froscio et al., 2003; Terao et al., 1994; Buratti et al., 2017). In addition, stomach, lungs, small intestine, thymus, and spleen can also be affected by histopathological lesions and cellular necrosis (Codd et al., 2019). In this regard, Terao et al. (1994) described the liver as the main target for CYN, which leads to protein synthesis inhibition, membrane proliferation, fat droplet accumulation, and cell death. This blockage, caused by the uracil moiety and the hydroxyl at C7 position, occurs at ribosomic level during the peptide chain elongation (Banker et al., 2001; Froscio et al., 2003; Machado et al., 2017). However, when CYP450 inhibitors were used, CYN toxicity decreased but no protection against the effect on protein synthesis was observed (Froscio et al., 2003; Buratti et al., 2017). Thus, the possible metabolites could exert toxicity through different mechanisms (Buratti et al., 2017).

- **Apoptosis** and morphological alterations were observed in HUVEC and Caco-2 cells (Gutiérrez-Praena et al., 2012a,b; Buratti et al., 2017).

- Increase of the **oxidative stress** biomarkers which, as a consequence, activate the p53 transcription factor, as described in cultured human dermal fibroblasts and HepG2 (Bain et al., 2007; Buratti et al., 2017). Cylindrospermopsin is thought to be the native toxin, as those cell lines do not present a significant metabolic activity (Buratti et al., 2017). Furthermore, intracellular formation of ROS was reported in HepG2 and human umbilical vein endothelial cell line (HUVEC), detecting in the last one an increase of c-glutamylcysteine synthetase and GSH content as well (Štraser et al., 2013c;

Gutiérrez-Praena et al., 2012, Buratti et al., 2017). In this sense, Runnegar et al. (1994; 2002) found a decrease of GSH in rat hepatocytes, which was attributed to the inhibition of GSH synthesis (Codd et al., 2016). Besides, a synergistic effect was observed after exposure to both CYN and a GSH inhibitor in the same experimental model (Soldatow et al., 2013). However, the results concerning the effects of CYN in GSH are not consistent. In this line, Ließel et al. (2011) did not find any alterations in the primary hepatocytes from the fish *Prochilodus lineatus*, while Gutiérrez-Praena et al. (2011) found a significant increase at low CYN concentrations but a reduction after exposure to high in fish PLHC-1 cells (Pichardo et al., 2017a). The depletion of GSH could be directly correlated to the increase in ROS levels and, these, to other toxic mechanisms, such as genotoxicity (Humpage et al., 2005; Pichardo et al., 2017a). However, this decrease on GSH levels could also be due to the protein-synthesis inhibition (WHO, 2020). The studies concerning other oxidative stress biomarkers such as lipoperoxidation or CAT are also varied (Pichardo et al., 2017a). Nonetheless, GSH reduction is not considered to contribute meaningfully to CYN acute toxicity *in vivo* (Norris et al., 2002; Pichardo et al., 2017a). In fact, when a toxicogenomic study was performed by Štraser et al. (2013c) on HegG2, only catalase (CAT) and thioredoxin reductase (TXNRD1) were upregulated, while NOS2, SOD1, or glutathione enzymes were not affected (Štraser et al., 2013c; Buratti et al., 2017).

- Connected with the oxidative stress damage, CYN has also been described as **pro-genotoxic** (Moreira et al., 2012; Pichardo et al., 2017b). This is based on its potentially reactive guanidine and sulphate groups (Shen et al., 2002; Humpage et al., 2000), favouring the interaction with nucleic acids (Bain et al., 2007; Pichardo et al., 2017b). The pro-genotoxic properties of CYN have been widely demonstrated in eukaryotic cells *in vitro* (Žegura et al., 2011; Puerto et al., 2018). In this sense, Humpage et al. (2005) and Bazin et al. (2010) demonstrated that CYP450 inhibitors such as omeprazole, SKF25A, or ketoconazole inhibited the genotoxicity induced by CYN alone, which indicates that the metabolism by CYP450 is needed for its genotoxicity (Humpage et al., 2005; Bazin et al., 2010). Furthermore, DNA damage was induced by CYN in HepG2 cells and human peripheral blood lymphocytes cells at non-cytotoxic concentrations, together with an upregulation of some CYP450 genes (Štraser et al., 2011; Žegura et al., 2011). However, when the Ames test was performed, no changes were observed after exposure to CYN with or without metabolic activation (Sierolawska, 2013; Buratti et al., 2017), which is in agreement with the results obtained in CHO-K1 cells by Fessard and Bernard (2003) and Lankoff et al. (2007). It is also relevant that CYN also led to an overexpression of the proteins involved in DNA repair, together with modifications of nucleosomal histones in Caco-2 cells (Huguet et

al., 2014; Buratti et al., 2017). In agreement, Štraser et al. (2013a,b) observed DNA damage oxidative stress-independent after exposure of HepG2 cells to non-cytotoxic concentrations of CYN. *In vivo*, CYN induces DNA-strand breakage in colon and bone marrow after oral administration to 1-4 mg/kg in mice after 24 hours (Bazin et al. 2012). However, the doses tested were quite high, which could induce cytotoxicity and thus, DNA damage (Buratti et al., 2017). Therefore, the genotoxicity potencial of CYN is still controversial (Buratti et al., 2017; Díez-Quijada et al., 2019c, 2020).

2. Pesticides

In addition to cyanotoxins, freshwater reservoirs can contain some pollutants able to cause environmental toxicity. In fact, the occurrence of harmful chemicals in the environment has become an issue of great concern in the last decades, being pesticides one of the most important groups (Bao et al., 2015; Damalas and Koutroubas, 2016).

Pesticides are chemical compounds designed to protect agricultural products from insects, undesirable plants, or fungal diseases, which led to a noticeable increase in crop yields and food production (WHO, 1996; Alexandratos and Bruisnma, 2012; Carvalho, 2017). These chemicals are applied annually in millions of tons of yields throughout the world (EPA 2017b; Dar et al., 2020), out of which 45% are applied in Europe and 25% in the USA (De et al., 2014; 2020). Despite being an agricultural advantage, their overuse leads to high toxicity, affecting soil, water, and natural resources where can be present, which leads to several environmental, animal, and human health issues (Toni et al., 2006; Bhardwaj et al., 2020). In this regard, a considerable fraction of the pesticides applied is dispersed into the environment, being found in every habitat, and are routinely detected in both aquatic and terrestrial animals (Vorley and Keeney, 1998; Law, 2014; Choudhary et al., 2018). Due to their perdurability, these compounds can be found after a long period, being able to exert toxic effects and disturb ecological balance by harming non-target organisms or causing genetic modifications that may lead to resistance (Andersson et al., 2014; Asghar et al., 2016). Based on their chemical structure, pesticides can be divided into organochlorines, organophosphorus, carbamates, pyrethroids, and neonicotinoids (Hakeem et al., 2017). From them, organophosphates (OPs) account for more than one third of the total pesticides globally consumed (Singh and Walker, 2006; Dar et al., 2020).

2.1. Organophosphates

These pesticides need to be metabolically activated via CYP450 through a desulfuration reaction to form an oxon (Hodgson and Rose, 2007; Richardson et al., 2019). These chemicals

are extensively applied in agriculture, horticulture, veterinary medicine, domestic purposes, and control of disease vectors (Dar et al., 2020). Their massive use is due to their high effectiveness against target pests and their relatively low toxicity to non-target organisms compared to other pesticides (Singh and Walker, 2006; Vijayalakshmi and Usha, 2012). Due to their widespread availability and their high-intensity use, OPs are associated with morbidity and mortality through occupational and accidental exposure or via suicidal attempts (Rohlman et al., 2011; Roman et al., 2019). General population may be exposed to pesticide residues in food and drinking water on a daily basis or to pesticide drift in residences close to spraying areas (Damalas and Eleftherohorinos, 2011; Damalas and Koutroubas, 2016). In this sense, according to the WHO, about 3 million people are exposed to OPs worldwide, leading to 300000 deaths each year (WHO, 2001; Roman et al., 2019). On the other hand, the treatment with OPs of agricultural products can lead to their discharge into the soil, leaching into groundwater, contaminating drinking water, and getting drift and thus, causing also air pollution (Dar et al., 2020).

In addition, OPs are mainly used due to their degradable organic nature and less persistence in the environment compared to other pests (Yang et al., 2005; Dar et al., 2020). However, despite their expected rapid degradation in aquatic systems, residual concentrations have been detected in water, soil, food, and human fluids, demonstrating their persistence for days/weeks and their bioaccumulation (Maurya and Malik, 2016; Carvalho et al., 1992; 2017). The bioaccumulation process usually is the consequence of the runoff from pesticide-contaminated agricultural land areas into the waterbodies such as streams, rivers, and oceans (Dar et al., 2020). Thus, these contaminants are ingested by fish and accumulate into the food chain, reaching humans (Maurya and Malik, 2016; Dar et al., 2020). Thus, OPs have been found in blood, urine, semen, breast milk, amniotic fluid, adipose tissue, and umbilical cord blood from humans (CSE report 2005; Kumar et al., 2016).

The main routes of exposure for OPs are the dermal route (by direct chemical contact), the oral route (ingestion of contaminated food or water), and inhalation (Sacramento, 2008; Dar et al., 2020).

2.1.1. Mechanisms of action

In general, all OPs present similar mechanisms of action, mainly affecting the synapsis:

- **Irreversible inhibition of acetylcholinesterase (AChE):** this inhibition occurs after phosphorylation of the hydroxyl group of the Serine 203 at the active site of the enzyme AChE (covalent bond), creating an AChE-OP complex. This leads to the accumulation of acetylcholine (Ach) at cholinergic synapses and neuromuscular junctions, causing a

disruption of the nervous impulse transmission at a synaptic level, leading to overstimulation (Kwong, 2002; Carvalho, 2017; Jokanovic, 2018). Death usually occurs as a consequence of respiratory failure from a combination of central and peripheral effects, paralysis of the respiratory muscles, and depression of the brain respiratory center (Karchmar, 2007; WHO, 1986; IPCS, 1998; Clark, 2002; Eyer, 2003; Marrs and Vale, 2006; Jokanović et al., 2011; 2018). The hazardous effects include impairment in normal body functioning, neurotoxicity, carcinogenicity, reproductive, and several other problems (Singh et al. 2007; Bhardwaj et al., 2020).

- **Overstimulation of nicotinic and muscarinic acetylcholine receptors (AChRs and mAChRs):** is a consequence derived from the AChE inhibition (Jokanović, 2018). The high stimulation of mAChR and nAChR disrupts the balance of glutamatergic, GABAergic, and cholinergic activities and changes the concentration of Ca^{2+} both inside and outside the cells (Aluigi et al., 2005; Grasshoff et al., 2003; Firozjaei et al., 2015). This change in Ca^{2+} concentrations affects ion concentration, hyperosmolarity, and protein function in the endoplasmic reticulum membrane, indicating possible prolonged effects on the structure and function of the brain (Angelini et al., 2004; Wade et al., 1987; McDonough and Shih, 1997; Piekut and Phipps, 1999; Firozjaei et al., 2015).

For 50 years, organophosphorus (OP) pesticides have been the most heavily and ubiquitously used worldwide, accounting for more than 50% of the total insecticide use worldwide being chlorpyrifos (CPF) on of special relevance (Burke et al., 2017; Rahman et al., 2021).

2.1.2. Chlorpyrifos

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is a broad-spectrum chlorinated organophosphate insecticide (Fig. 9) used for pest control on crops such as corn, citrus fruits, cotton, nuts, beets, legumes, potatoes, etc. (Alizadeh et al., 2018), lawns and ornamental plants, being detected in most of the countries worldwide (Lai, 2020; John and Shaike, 2015). Its overuse is due to its low persistence, cost-efficiency, wide-ranging applicability, and low range of toxicity (Rahman et al., 2021). In this regard, CPF is registered for its use in nearly 100 countries and is annually applied to 8.5 million crops acres (Mukti et al., 2018; Koly and Khan, 2018). Furthermore, this pesticide is the most used one in the European Union (1226 tons, Eurostat) and one the most common in the United States (8-11 million pounds applied in 2007 (US EPA, 2016; Lai, 2020). This excessive and continuous use in agriculture led to the persistence of CPF residues in various environmental compartments, such as soil, water, and air, being

detected even at 24 kilometers from the application site (Vijayalakshmi and Usha, 2012; Koly and Khan, 2018). However, this OP has been recently removed from the list of allowed chemicals in EU (EFSA, 2019).
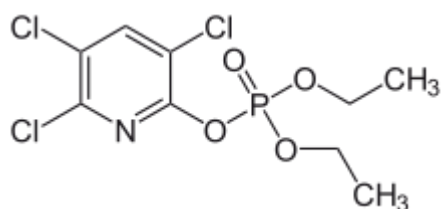

Figure 9. CPF chemical structure.

In general, CPF enters the soil environment by direct pesticide application, leading to its detection in edibles, sediments, streams, sumps, reservoirs, rivers, sea, urban storm drains, freshwater lakes, groundwater, ice, snow, fog, rain and air in many countries, including the artic regions (Pengphol et al., 2012; Watts, 2012). However, its absortion depends on many environmental factors (John and Shaike, 2015). The half-life of CPF in soil generally depends on many factors such as soil microorganisms, application rate, pH, moisture content, pesticide formulation, organic carbon content, and climatic conditions (John and Shaike, 2015). However, its persistence has been shown to vary from a few days up to 4 years (Rahman et al., 2021).

Chlorpyrifos has been demonstrated to exert acute toxicity in insects, birds, mammals, and aquatic life, by the affectation of the CNS as well as cardiovascular, endocrine, and respiratory systems in non-target organisms (Kavitha and Rao, 2008; Shafiq-Ur-Rehman and Waliullah, 2012). In general, in target and non-target organisms, acute exposure to CPF can lead to tingling sensation, numbness, dizziness, vomiting, sweating, nausea, stomach cramps, headache, incoordination, muscle twitching, vision disturbances, slurred speech, drowsiness, confusion, anxiety, depression and, in worst cases, respiratory arrest, unconsciousness, convulsions, and even death (Rayu et al., 2017).

The main routes of exposure for CPF in humans are through contact, ingestion, and inhalation, as 70% of the absorption is by oral route and less than 3% through dermal contact (Alizadeh et al., 2018). In this sense, the exposure to CPF occurs mainly through contaminated environment and dietary intake (Saunders et al., 2012; Todd, 2017).

2.1.2.1. Toxicity of CPF

The liver is the main responsible for CPF metabolism, being usually excreted without bioaccumulation through urine within 24 hours (Griffin et al., 1999; Wagner et al., 1999; Lai, 2020; Alizadeh et al., 2018). However, animals convert CPF to its oxidized forms "oxon", which

is about 3000 times more potent than the parent compound in the nervous system (Chambers and Carr, 1993; Sultatos, 1991; Koly and Khan, 2018).

The mechanism of action of CPF-oxon, which is generated through oxidative desulfuration *in vivo* is the same as the rest of OPs after acute exposure: binding to AChE in the active site of Serine 203, inhibiting it irreversibly, leading to the accumulation of ACh, and thus, causing a serious disruption of nervous activities (Chambers and Chambers, 1989; Eaton et al., 2008; Todd, 2017). This inhibition leads to seizures, paralysis and, in worst cases, death of insects and mammals (Alizadeh et al., 2018). In this regard, some animal studies have demonstrated that CPF may lead to anxiety-like behavior (Chen et al., 2011; Hashjin et al., 2013; López-Crespo et al., 2007; Roman et al., 2019). In humans, farmers exposed to CPF have been demonstrated to have a higher risk of developing symptoms related to anxiety and mood disorders, even after low-level exposure (Harrison and Mackenzie Ross, 2016; Koh et al., 2017; Roman et al., 2019). Furthermore, CPF has been demonstrated to interact with macromolecules synthesis (DNA, RNA, and proteins), neurotransmitter receptors and signal transduction pathways (Koly and Khan, 2018). In addition, this pesticide impedes respiration in the livers of laboratory animals by interacting with the activity of ATPase and cholesterolester-hydrolase, which are associated with cellular respiration and normal reactions to stress respectively (Civen et al., 1977; Sakai, 1990; Koly and Khan, 2018). The main mechanisms of CPF toxicity are summarized in Fig. 10.
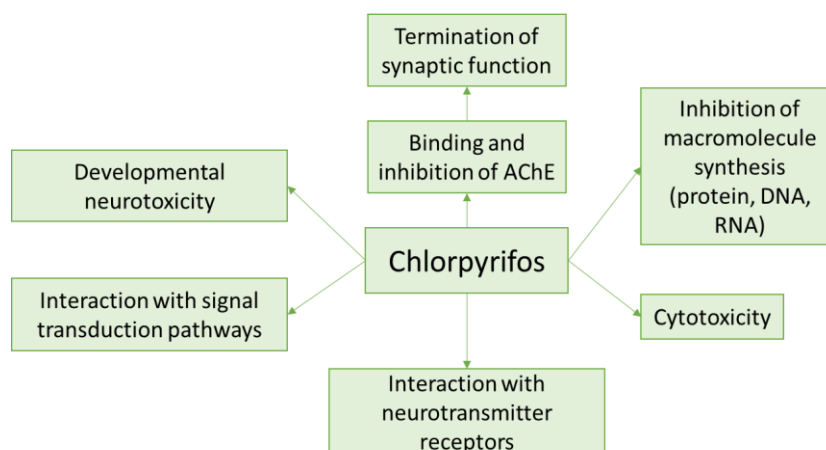
Figure 10. Mechanisms of CPF toxicity

Moreover, chronic exposure to CPF has also been demonstrated to cause low birth weight, birth defects, and immune and endocrine systems impairments in non-target organisms (Alizadeh et al., 2018). In this sense, there are many studies concerning CPF toxicity in several fish species that include unusual swimming behavior, paralysis, histological abnormalities, and reduced survival, adult length and body weight together with reproductive failure. In addition, structural alterations and biochemical changes such as ion transport alteration, lipid peroxidation, depletion in protein content, decrease of different metabolic enzyme levels, hormonal changes, mutagenic

and genotoxic effects have also been reported (Farag et al., 2003; Tian et al., 2005; Slotkin et al., 2005; De Angelis et al., 2009; Haviland et al., 2010; Da Silva and Samayawardhena, 2002; Sharbidre et al., 2011; John and Shaike, 2015).

Concerning the effects of CPF in humans, both acute and chronic exposures to this pesticide led to genotoxicity and mutagenicity (Rahman et al., 2021). According to epidemiological studies, CPF exposure can be linked to increased risk of colorectal, prostate, breast, lymphoma, hematopoietic, leukemia, and brain cancers (Lee et al., 2004; 2007; Alavanja et al., 2003; Engel et al., 2005; Karunanayake et al., 2012). Furthermore, it has endocrine implications, such as anti-androgenic and estrogenic properties, and alterations in thyroid and adrenal glands (Usmani et al., 2003; Viswanath et al., 2010; Meeker et al., 2008; Ventura et al., 2012; Fortenberry et al., 2012). Chlorpyrifos is also associated with reproductive factors such as DNA damage, decrease in the fluid, concentration, and motility of sperm, birth weight, length problems, cervical fluid, cord blood, meconium, and breast milt (Watts, 2012; John and Shaike, 2015). Additionally, due to its lipophilic properties, CPF can easily cross the placenta, being able to exert toxicity in the fetus (Akhtar et al., 2006; Todd, 2017). In this regard, subtoxic doses of CPF are able to affect brain development by altering neuronal activity and reactivity and by inhibiting mitosis, and thus, inducing apoptosis (John and Shaike, 2015). In general, prenatal exposure to CPF is associated with the reduction in fetal growth indices (Eskenazi et al., 2004; Whyatt et al., 2004; Todd, 2017), cognitive functions (Perera et al., 2003; Rauh et al., 2006; 2011) and the integrity of the brain structure (Rauh et al., 2012; Todd, 2017). Gestational exposure to CPF is associated with developmental delay and autism spectrum disorders (ASD), increasing the diagnosis of ASD by 60% in children born from mothers living close to a CPF application site during gestation (Shelton et al., 2014; Todd, 2017). Deficits in the number of neural cells, suppression of gene expression, and macromolecular differentiation factors, with subsequent abnormalities of synaptic function and behavioral performance after repeated exposure to CPF to concentrations that did not cause AChE inhibition have been also reported (Dam et al., 1998; 2000; 2003; Campbell et al., 1997; Johnson et al., 1998; Levin et al., 2001; Slotkin, 2001a; 2002b; 2004). However, CPF has demonstrated to interact with Serine hydrolases, muscarinic receptors, cannabinoid receptors, and structural proteins, which play critical roles in neuronal differentiation, synaptogenesis, neuritic outgrowth, and modulation of synaptic transmission (Huff et al., 1994; Jett and Lein, 2006; Terry, 2012; Crumpton et al., 2000; Dam et al., 2003; Garcia et al., 2001). Furthermore, free, circulating CPF can bind to butyrylcholinesterase (BuChE) (Doctor and Saxena, 2005; Todd, 2017), which is a bioscavenger for OP pesticides and is commonly used as a biomarker of OP exposure (Gazzi et al., 2014; Jalady and Dorandeu, 2013; Strelitz et al., 2014). However, the levels of BuChE in adults are four times more than at birth

(Sidell, 1992; Todd, 2017), reducing the amount of CPF bioscavenged by BuChE and affecting the fetal and children sensitivity to CPF (Moser et al., 1998; Pope et al., 1993; Todd, 2017).

Exposure to high levels of CPF in children led to delays in psychomotor and mental development, attention deficit or hyperactivity disorder, and developmental disorder problems (Rauh et al., 2006; John and Shaike, 2015; EPA, 2016). In addition, CPF is also thought to impact neurological functions by the gut-brain axis, where a dysbiosis can result in disorders at CNS and gastrointestinal levels (Carabotti et al., 2015; Lai, 2020). According to epidemiological data, children present higher blood and urine levels of CPF than adults, probably due to their lower detoxification capacity and the smaller body surface area, together with their habits (Barr et al., 2015). Concerning adults, although most environmental exposures to CPF do not seem to induce overt signs of acute intoxication, some epidemiological studies point out CPF as a possible cause for cognitive deficits in healthy adults (Kaplan et al., 1993; Steenland et al., 2000; Todd, 2017).

3. Combinational studies and their importance

As chemicals can change their toxic properties depending on the characteristics of their surroundings, studying toxicants as they are present in nature is of great concern (Walker, 1998). Among the different possible scenarios, the presence of more than one chemical in nature can lead to effects that cannot be produced by the compounds alone (WHO, 2017). In this regard, the studies on simultaneous and sequential exposure to toxins, and to toxin mixtures, could lead to the assessment of additive, synergistic or antagonistic effects, which is especially important in the case of CYN, as this cyanotoxin is usually found in the presence of other toxics such as metals, microplastics, pesticides or other cyanotoxins (Metcalf and Codd, 2020). In fact, CYN has been reported to act synergistically with other cyanotoxins, causing mass mortality (Yang et al., 2021).

Taking into account that MC-LR and CYN are two of the most common cyanotoxins and that their concomitance has been demonstrated in different countries worldwide, the effects of their combination is of importance (Bittencourt-Oliveira et al., 2014; Testai et al., 2016). The environmental and health impact of the combination of known cyanotoxins with different mechanisms of action has scarcely been investigated (Metcalf and Codd, 2012). However, the production of different cyanotoxins from the same strains or the combination of mixed cyanobacterial blooms are common scenarios and sometimes are the cause of some intoxication cases (Metcalf and Codd, 2012). There have been many cases of animal and human intoxication, apart from the natural fish-kill, because of cyanobacterial life cycles, which have led to a better understanding of the ecotoxicology of cyanotoxins (Metcalf and Codd, 2012).

Pesticides and cyanobacterial products have also demonstrated to be present together in both aquatic and terrestrial environments, leading to a possible interaction between them (Singh et al., 2018). The literature regarding the combination of pesticides and cyanotoxins is very scarce, although this combination has demonstrated to be present in nature, being the case of the decrease of American alligator (*Alligator mississippiensis*) in Florida associated with interactions between cyanotoxins and pesticides (Woodward et al., 2011). For this reason, the study of the combination of CYN and CPF is of interest.

4. Neurotoxicity induced by cyanotoxins and pesticides

Despite being classified as hepatotoxins, MC-LR and CYN have been demonstrated to cause toxicity in many organs. In the case of MC-LR, the studies concerning its neurotoxic properties constitute a solid base to consider this cyanotoxin. In this regard, this cyanotoxin has demonstrated its capacity to cross the blood-brain-barrier through the OATPs transporters, being detected in the brain of several experimental models. The main neurotoxic effects seem to be related to histopathological and biochemical changes in brain, mainly oxidative stress and inhibition of PPs, together with behavioral alterations.

In the case of CYN, it has also demonstrated some neurotoxic effects, although the studies regarding this are still scarce. In this sense, the main effects observed were inflammation, apoptosis, oxidative stress, and alteration of AChE.

Additionally, CPF is a pesticide designed to affect the nervous system of insects, although it has also been demonstrated to cause neurotoxicity in humans. However, the effects produced by this chemical in concomitance with cyanotoxins have not been studied so far.

Due to all the explained above, it is important to study the effects of these toxicants in the nervous system, isolated, but also in combination with other compounds that are likely to be present in the same environmental samples when the organisms are exposed.

II. SIGNIFICANCE AND PURPOSES

1. Significance

Taking into account the previously mentioned, it would be of interest to study the effects that the combinations of CYN + MC-LR and CYN + CPF could exert in the human nervous system, together with the neurotoxic properties of CYN, using for this purpose the human neuronal cell line SH-SY5Y and murine primary neuron cultures.

The **SH-SY5Y cell line** is one of the most commonly used experimental models among all the available for neurotoxicity studies (Todd, 2017). This cell line is a subclone of SK-SN-SH neuroblastoma cell line isolated from a bone marrow of a 4-year-old female patient, which possesses a stable karyotype of 47 chromosomes (Shipley et al., 2016, 2017). One of the most important characteristics of this immortal cell line is its capacity to differentiate, resembling, chemically and morphologically, different mature neuronal populations (Påhlman et al., 1995; Shipley et al., 2016; Todd, 2017). Furthermore, the SH-SY5Y cell line presents a basal expression of neurotransmitters, essential for dopaminergic and cholinergic signaling, making this cell line a really interesting experimental model for the study of the Parkinson's or Alzheimer's diseases, among others (Agholme et al., 2010; Kovalevich et al., 2013; Xicoy et a., 2017; Xie et al., 2010; Todd, 2017).

Undifferentiated SH-SY5Y cells proliferate rapidly and are morphologically characterized as neuroblast-like non-polarized cells, expressing immature neuronal markers (Kovalevich and Langford, 2013; Shipley et al., 2016; Todd, 2017). In this regard, these cells are considered most reminiscent of immature catecholaminergic neurons (Kovalevich and Langford, 2013). However, during differentiation, the proliferation rate decreases, and cells start to express mature neuronal protein markers (Shipley et al., 2016). In addition, the differentiation process leads to the formation and extension of neuritic processes, electrical excitability of the plasma membrane, formation of synaptophysin-positive functional synapses, expression of various neurotransmitters, and neuron-specific enzymes (Kovalevich and Langford, 2013). When fully differentiated, some of the markers of mature neurons appear in this cell line: neuronal nuclei (NeuN), synaptophysin (SYN), growth-associated protein (GAP-43), neuron-specific enolase (NSE), synaptic vesicle protein II (SV2), and microtubule-associated protein (MAP), among others (Gimenez-Cassina et al., 2006; Påhlman et al., 1984; Xie et al., 2010; Cheung et al., 2009; Shipley et al., 2016). Furthermore, the removal of the trophic factor BDNF leads to cellular apoptosis, as occurs in mature neurons (Encinas et al., 2000; Shipley et al., 2016). There are different procedures to differentiate cells according to the subtype of interest according to the specific neuronal subtype population desired. In this respect, RA is a vitamin A derivative known for causing inhibition of cellular growth and differentiation-promoting properties (Kovalevich and Langford, 2013). This differentiation leads to a cholinergic neuron phenotype, with is

evidenced by the increase in the choline acetyltransferase (ChAT) activity expression and vesicular monoamine transporter (VMAT) expression (Lopes et al., 2010; Presgraves et al., 2004; Kovalevich and Langford, 2013). Furthermore, the expression of both mAChRs and nAChRs have been reported in SH-SY5Y cells differentiated with RA (Kovalevich and Langford, 2013).

The **murine neuronal primary cultures** are also an important experimental model for neurotoxicity and developmental neurotoxicity studies, as they are real neurons. Thus, these cultures allow the examination of the activity and properties of neurons at cellular and synaptic levels (Nunez, 2008). In addition, the main difference between cell lines and primary cultures is that the primary cultures do not derive from tumours, which makes of these cells a more interesting cell model in terms of neuronal properties *in vivo*. However, this type of culture is hard to maintain, as mature neurons do not proliferate. Furthermore, it is required to separate the cell type of interest from the rest, such as glial cells (Gordon et al., 2013).

Both differentiated SH-SY5Y and primary cultures express biomarkers of interest. In this sense, the NeuN protein is a commonly used marker to identify post-mitotic neurons, as is expressed exclusively in nervous mature cells (Todd, 2017). In addition, the MAP-2 is a protein that stabilizes dendrites in post-mitotic neurons (Soltani et al., 2005). The expression of this protein depends on growth factors and neurotransmitters, and also appears during cell proliferation and differentiation, although not only in neuronal tissues (Johnson and Jope, 1992; Todd, 2017). Concerning the synapsis, synaptophysin and postsynaptic density protein 95 (PSD95) are pre- and postsynaptic proteins, respectively. Synaptophysin is an integral membrane protein of small synaptic vesicles and is found in all nervous tissue, while PSD95 is a synaptic protein that interferes in the synaptic maturation by interacting with several receptors of the postsynaptic membrane (Glantz et al., 2008; Coley and Gao, 2018). Both proteins are of great interest for investigating the neuronal synaptic function.

2. Purposes

Therefore, the specific aims established in the present Doctoral Thesis have been:

1. To perform a comprehensive literature review of the studies performed *in vitro* and *in vivo* concerning the neurotoxic effects of MCs and CYN.
2. To assess the effects of MC-LR, the most abundant MCs variant, and CYN in cell viability, oxidative stress generation, AChE activity, and morphological alterations, alone and in combination, in undifferentiated and differentiated SH-SY5Y cells.

3. To study the effects of CPF isolated and in combination with CYN, in the undifferentiated and differentiated SH-SY5Y cell line concerning viability, oxidative stress parameters, AChE activity and morphological alterations.

4. To evaluate the effects of a CYN-producer and non-producer cyanobacterial extracts in the SH-SY5Y cell line using viability and oxidative stress as biomarkers.

5. To study the effects of pure CYN in murine primary cultures in terms of viability and synapsis by immunocytochemistry.

6. To study the effects of CYN, CPF and their combination in the nAChRs in order to assess a possible mechanism of action acute toxicity in 3 days-differentiated SH-SY5Y. Furthermore, studies concerning the effect on developmental neurotoxicity of these toxics in SH-SY5Y during 6 days of differentiation by assessing their effect on cell viability and neurite outgrowth.

The experimental work for aims 1-6 h was performed at the Area of Toxicology of the Faculty of Pharmacy of the University of Sevilla, using the infrastructures from the Biology and Microscopy Services of the Centro de Investigación, Tecnología e Innovación of the University of Sevilla (CITIUS) as support. The morphological analysis was performed in collaboration with Dr. Francisco Javier Moreno, from the Ultrastructural Cytochemistry group of the Department of Cellular biology from the Faculty of Biology of the University of Sevilla. The experimental work for aim 7 was performed at the Department of Biochemistry and Biophysics of the Stockholm University, under the supervision of Dr. Anna Forsby.

Furthermore, an internship for 7 months that led to a contract for 10 more months was performed in Anna Forsby's group. In this sense, one research work has been published in collaboration with the EU-Tox Risk project. In addition, according to the regulations from the University of Sevilla, this thesis has been completely written in English to fulfill the requirements to aim the PhD with International Mention.

III. RESULTS

CHAPTER 1

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Francisco Javier Moreno, Ana María Cameán, Ángeles Jos

NEUROTOXIC ASSESSMENT OF MICROCYSTIN-LR,

CYLINDROSPERMOPSIN AND THEIR COMBINATION ON THE

HUMAN NEUROBLASTOMA SH-SY5Y CELL LINE

Neurotoxic assessment of Microcystin-LR, cylindrospermopsin and their combination on the human neuroblastoma SH-SY5Y cell line

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A R T I C L E   I N F O

A B S T R A C T

Microcystin-LR (MC-LR) and Cylindrospermopsin (CYN) are produced by cyanobacteria. Although being considered as a hepatotoxin and a cytotoxin, respectively, different studies have revealed neurotoxic properties for both of them. The aim of the present work was to study their cytotoxic effects, alone and in combination, in the SH-SY5Y cell line. In addition, toxicity mechanisms such as oxidative stress and acetylcholinesterase (AChE) activity, and morphological studies were carried out. Results showed a cytotoxic response of the cells after their exposure to 0–100 μg/mL of MC-LR or 0–10 μg/mL CYN in both differentiated and undifferentiated cells. Thus, CYN resulted to be more toxic than MC-LR. Respect to their combination, a higher cytotoxic effect than the toxins alone in the case of undifferentiated cells, and almost a similar response to the presented by MC-LR in differentiated cells were observed. However, after analyzing this data with the isobolograms method, an antagonistic effect was mainly obtained. The oxidative stress study only showed an affectation of glutathione levels at the highest concentrations assayed of MC-LR and the combination in the undifferentiated cells. A significant increase in the AChE activity was observed after exposure to MC-LR in undifferentiated cells, and after exposure to the combination of both cyanotoxins on differentiated cells. However, CYN decreased the AChE activity only on differentiated cultures. Finally, the morphological study revealed different signs of cellular affectation, with apoptotic processes at all the concentrations assayed. Therefore, both cyanotoxins isolated and in combination, have demonstrated to cause neurotoxic effects in the SH-SY5Y cell line.

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1. Introduction

Cyanobacteria are present in a variety of aquatic and terrestrial ecosystems due to their adaptive ability, even in extreme conditions (Svirčev et al., 2014). Under favorable conditions of light, pH, nutrients (nitrogen and phosphorus) and interaction with other organisms, they present the capability of forming blooms and producing secondary metabolites called cyanotoxins, whose occurrence is increasing due to long term climate change (Buratti et al., 2017). According to their target organ, these toxins can be classified as hepatotoxins (e.g. microcystins, nodularins), dermatotoxins (e.g. lungbyatoxin), neurotoxins (e.g. anatoxin-a, homoanatoxin, saxitoxins), irritant toxins (e.g. lipopolysaccharides) and cytotoxins (e.g. cylindrospermopsin) (Testai et al., 2016). The exposure to these metabolites can occur by different paths such as the oral route, dermal contact or inhalation, although the oral route is the most significant one, since intoxication may take place by the intake of contaminated water, food or dietary supplements based on algae (Buratti et al., 2017). Among all cyanotoxins, microcystins (MCs) and cylindrospermopsin (CYN) have focused great interest, since they have been involved in the death of different animal species and humans (Azevedo et al., 2002;

Bourke et al., 1983; Carmichael et al., 2001; Malbrouck and Kestemont, 2006).

Microcystins are cyclic heptapeptides synthetized by several cyanobacterial species such as *Mycrocistis aeruginosa, Oscillatoria agardhii, Plankthotrix agardii*, and *Planktothris rubescens,* etc. (Sivonen and Jones, 1999). Up to date, more than 246 congeners of MCs are known, being MC-LR the most potent congener and frequently identified (Spoof and Catherine, 2017). Mainly considered as a hepatotoxin, MC-LR can also affect other organs such as kidneys, heart or brain (Li et al., 2012; Qiu et al., 2009; Zeng et al., 2014, Zhang et al., 2018). One of the main MC-LR-mechanisms of action is the inhibition of protein serine/threonine phosphatases, causing a cascade of effects such as the deregulation of phosphoproteins, which lead to tumor promotion and apoptosis (MacKintosh et al., 1990). In addition, many studies have also demonstrated its *in vitro* cytotoxic potential in different cell lines from fish, mammals and humans (Ding et al., 2017; Feurstein et al., 2009; Gutiérrez-Praena et al., 2012; Meng et al., 2011, 2013; Pichardo et al., 2005; Rozman et al., 2017). Moreover, several authors have also described that this toxin induces oxidative stress by increasing reactive oxygen species (ROS) and reducing glutathione (GSH) levels, leading to cell apoptosis (Li et al., 2015; Liu et al., 2016; Puerto et al., 2011; Qian et al., 2018), although these effects have not been studied using human neuronal cell lines yet.

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Cylindrospermopsin is an alkaloid consisting in a tricyclic guanidine combined to a hydroxylmethyl uracil group. This toxin presents a highly water-soluble structure, being commonly found out of the cells (Falconer and Humpage, 2006). Several cyanobacterial species are able to produce this toxin, such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Chrysosporum ovalisporum, Anabaena bergii,* etc. (Banker et al., 1997; Harada et al., 1994; Schembri et al., 2001; Shaw et al., 1999). The main target of this cytotoxin is the liver, although kidneys, lungs, thymus, marrow bone, adrenal gland, gastrointestinal tract, immune, heart and nervous system have been also described as potential targets (Falconer, 1999; Guzmán-Guillén et al., 2015; Hawkins et al., 1985; Humpage et al., 2005; Terao et al., 1994). The most well-known mechanism of action of CYN is the inhibition of protein and GSH synthesis (Froscio et al., 2003; Runnegar et al., 1995; Terao et al., 1994). This cyanotoxin also enhances ROS production, which could lead to apoptosis or DNA damage (Gutiérrez-Praena et al., 2011, 2012; Guzmán-Guillén et al., 2013; Puerto et al., 2011). In addition, some studies have indicated the pro-genotoxic properties of CYN, being essential its previous metabolic activation by the enzymatic cytochrome P-450 complex (CYP450) (Humpage et al., 2005; Puerto et al., 2018; Žegura et al., 2011).

Both cyanotoxins have evidenced to induce neurotoxic effects in different experimental models (Florzyk et al., 2014). Thus, MCs have shown to cause neuronal damage *in vitro* in different rodent cell lines such as primary murine cerebellar granule neurons (CGNs) and primary rat astrocytes (Feurstein et al., 2011; Rozman et al., 2017). Furthermore, many *in vivo* studies have manifested a clear neurotoxic potential, mostly of MC-LR, in different animal species such as rodents, fish and nematodes, affecting to their behavior, enhancing ROS levels, and modifying proteins related to neurodegenerative diseases (Baganz et al., 2004; Wang et al., 2013; Wu et al., 2017). In fact, MC-LR has produced pathological damage in hippocampus, neuronal degenerative changes, inflammation in memory-related brain regions and apoptosis in rats, suggesting that this toxin can be related to Alzheimer's disease in humans (Li et al., 2012; 2014). Actually, some studies confirm its transport through the blood-brain-barrier using OATP1A2, a variant of the organic anion transport system (OATP), because of its relatively large hydrophilic structure (Feurstein et al., 2009; Fischer et al., 2005; Menezes et al., 2013). On the contrary, neurotoxic effects of CYN are still not well elucidated. In this sense, there is only one report studying its effects in different *in vitro* murine cell lines (Takser et al., 2016). Meanwhile, some *in vivo* reports have showed *in vivo* neurotoxic effects in snails, tadpoles and fish, such as behavioral alterations or histopathological changes (Da Silva et al., 2018; Guzmán-Guillén et al., 2015; Kinnear et al., 2007; Kiss et al., 2002; White et al., 2007).

Moreover, it is worthy to point out that the majority of studies concerning cyanotoxins toxicity are focused on single purified toxins, setting apart the fact that organisms are exposed simultaneously to a wide variety of cyanotoxins when they are present in aquatic systems. In fact, several studies have described the concomitant occurrence of MCs- and CYN-producing cyanobacteria, as well as the presence of both cyanotoxins at the same time (Bittencourt-Oliveira et al., 2014; Bogialli et al., 2006; Oehrle et al., 2010; Vasas et al., 2004). In the case of neurotoxicity, only a study conducted by Takser et al. (2016) showed the effects of a combination of MCs and CYN, although they also included the neurotoxin anatoxin-a in the combination (1:1:1).

The SH-SY5Y cell line is a commonly used neuronal model due to its biochemical and functional properties, being very appropriate for neurotoxicity studies. Moreover, the differentiation of this cell line provides functional, biochemical and morphologically mature neurons, which are more similar to those present in the human brain

(Xie et al., 2010). For this reason, both types of SH-SY5Y cells are a very interesting experimental model to assess the possible damage induced by MC-LR and CYN in human neural cells.

Thus, considering all this, the aim of the present study was to assess the neurotoxic potential of MC-LR, CYN and their combination *in vitro* using the human neuroblastoma SH-SY5Y cell line, by exploring the cell viability, oxidative stress (ROS and GSH levels), acetylcholinesterase (AChE) activity and morphological changes after the exposure to these cyanotoxins.

2. Materials & methods

2.1. Supplies and chemicals

MC-LR and CYN (both purity > 95% by HPLC) were purchased from Enzo Life Sciences. Minimum essential medium (MEM), cell culture reagents, and fetal bovine serum (FBS) were obtained from Gibco (Biomol, Sevilla, Spain). Nutrient Mixture F-12 Ham, retinoic acid (RA), and brain-derived neurotrophic factor human (BDNF) were purchased in Sigma-Aldrich (Madrid, Spain).

The MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulphophenyl)-2H-tetrazolium salt) Cell Titer 96® AQueous One Solution Cell Proliferation Assay was purchased in Promega (Biotech Iberica, Madrid, Spain). The Bradford Reagent and the neutral red (NR) were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Model system

SH-SY5Y cells derived from a human neuroblastoma were obtained from ATCC (CRL-2266). They were maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain) in a medium consisting in MEM and F-12 (1:1) supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine 200 mM, and 1% penicillin/streptomycin solution. Cells were grown near confluence in 75-cm² plastic flasks and harvested weekly with 0.25% trypsin-EDTA (1X). Cells were quantified in a Neubauer chamber. SH-SY5Y cells were plated at density of $2 \cdot 10^5$ cells/mL to perform all the experiments.

2.3. Cell differentiation

SH-SY5Y cells were differentiated using the protocol provided by Encinas et al. (2000) with some modifications. Cells were plated at density of $5 \cdot 10^2$ cells/mL in plates of 48 wells, changing the medium every 48 h with 1% of FBS, 10 μM RA and 50 ng/mL BDNF, for a week. The differentiation process was evaluated by morphological analysis.

2.4. Toxin test solutions

Stock solutions of 4 mg/mL MC-LR and 1 mg/mL CYN were prepared in absolute ethanol and sterilized milliQ water, respectively. Both solutions were maintained at −20 °C until their use.

2.5. Cytotoxicity assays

Undifferentiated SH-SY5Y cells were seeded in 96-well tissue-culture plates for basal cytotoxicity tests and incubated at 37 °C for 24 h prior to exposure. Differentiated cells were exposed after a week from the start of the differentiation process in the same 48-well plates where the differentiation process took place. From the stock solution

of MC-LR, serial dilutions in medium without serum were prepared (20, 40, 60, 80, 100 μg/mL MC-LR). From the stock solution of CYN, serial dilutions in medium without serum were prepared (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2.5, 5, 10 μg/mL CYN). Vehicle control (ethanol) for MC-LR and a negative control (non-treated cells) were also included. After replacing the medium, exposure solutions were added to the plates, and incubated at 37 °C for 24 and 48 h. The basal cytotoxicity endpoints assayed were protein content (PC), supravital dye neutral red cellular uptake (NR), and tetrazolium salt reduction (MTS). All the assays in the present paper were performed by triplicate.

Total protein content (PC) was quantified *in situ*, according to the procedure given by Bradford (1976), with modifications (Pichardo et al., 2005), in the same plates where exposure originally took place. The culture medium was replaced by 200 μL NaOH to dissolve the proteins and after 2 h of incubation at 37 °C, 180 μL were replaced by the same volume of Bradford reagent. After 30-min incubation at room temperature, absorbance was read at 620 nm.

Neutral red uptake was performed according to Borenfreund and Puerner (1985) in the undifferentiated cells. The culture medium was replaced by 100 μL modified medium without serum containing 10 mg/mL NR. The plate with the NR-containing medium was returned to the incubator for another 3 h to allow the uptake of NR into the lysosomes of viable cells. Thereafter, medium was removed, and cells were fixed for 2 min with a formaldehyde–CaCl₂ solution. By adding 200 μL of acetic acid–ethanol solution to the wells, NR absorbed by cells was extracted, solubilized, and quantified at 540 nm.

The MTS reduction was measured according to Barltrop et al. (1991) in both undifferentiated and differentiated cells. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) tetrazolium compound was added to the medium and, by bioreduction of cells, produces a colored formazan product soluble in culture medium, which is immediately measured at 490 nm after 3 h of incubation in the dark.

2.6. Assessment of the effect of cyanotoxins combination by the isobolograms method

Concentrations used to evaluate the toxic potential of the combination MC-LR - CYN were selected based on the cytotoxicity results of the single cyanotoxins previously obtained in both types of SH-SY5Y cells. The mean effective concentration (EC₅₀) values obtained for the most sensitive endpoint at 24 h were chosen as the highest exposure concentrations for the combination studies, along with the fractions EC₅₀/2 and EC₅₀/4. Thus, SH-SY5Y cells were exposed for 24 and 48 h to binary pure cyanotoxins combinations: EC₅₀ MC-LR + EC₅₀ CYN, EC₅₀/2 MC-LR + EC₅₀/2 CYN and EC₅₀/4 MC-LR + EC₅₀/4 CYN, and the MTS reduction assay was performed. Moreover, each concentration used in the combinations was evaluated for each individual cyanotoxin, also using the MTS assay.

The isobologram method was used to determine the type of interaction that occurs when MC-LR and CYN are in combination in undifferentiated and differentiated SH-SY5Y cells. This method was carried out according to Tatay et al. (2014). The isobologram analysis involves plotting the concentration-effect curves for each compound and its combinations in multiple diluted concentrations by using the median-effect equation, as described by Chou and Talalay (1984) and Chou (2006). These authors introduced the term of combination index (CI) for the quantification of synergism, additivity or antagonism of two compounds. When the CI < 1, indicates synergism, when CI is = 1, indicates additivity, and when CI is > 1, indicates antagonism. The CI₅₀, CI₇₅ and CI₉₀ are the concentrations required to inhibit pro-

liferation at 50%, 75% and 90%, respectively. The CalcuSyn software (version 2.1) calculated these CI values automatically (Biosoft, Cambridge, UK, 1996–2007). The type of interaction produced by MC-LR and CYN combinations was assessed by an isobologram analysis using the same software. The parameters *Dm*, *m*, and *r* of the combinations, are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, respectively, and they give information about the shape of the concentration–effect curve.

2.7. Oxidative stress assays

2.7.1. Reactive oxygen species (ROS) generation

The production of ROS was assessed in 96-well plates using the dichlorofluorescein (DCF) assay (Puerto et al., 2010) in undifferentiated cells. Cells were incubated with 200 μL 40 μM DCF in the culture medium at 37 °C for 30 min. Then, cells were washed with PBS and exposed to the different concentrations of the toxins, according to the cytotoxicity results previously obtained (0–37 μg/mL MC-LR and 0–1 μg/mL CYN). A solution of 200 μM MnCl₂·4H₂O was used as a positive control. The plates were incubated for 4, 8, 12 and 24 h. Fluorescence was measured at 535 nm (emission) and 485 nm (excitation).

2.7.2. Glutathione (GSH) content

Glutathione (GSH) content was evaluated by reaction with the fluorescent probe monochlorobimane (mBCL) (Jos et al., 2009) in undifferentiated cells. Cells were exposed to the toxins (0–37 μg/mL MC-LR and 0–1 μg/mL CYN), according to the previous results obtained in the cytotoxicity assays and incubated for 4, 8, 12 or 24 h. A solution of 1 μM buthionine sulfoximine (BSO), a GSH synthesis inhibitor, was used as positive control. After the exposure time, medium was discarded and cells were incubated for 20 min at 37 °C in the presence of 40 μM mBCL. After that, cells were washed with PBS and the fluorescence was measured 460 nm (emission) and 380 nm (excitation).

2.8. Acetylcholinesterase (AChE) activity determination

Acetylcholinesterase activity was measured according to the method described by Ellman et al. (1961) with modifications of Santillo and Liu, (2015) in both undifferentiated and differentiated cells. Viable SH-SY5Y cells were exposed to the toxins, according to the previous results provided by the cytotoxicity assays (0–37 μg/mL MC-LR and 0–1 μg/mL CYN in undifferentiated cells; 0–45 μg/mL MC-LR and 0–0.3 μg/mL in differentiated cells) and incubated for 24 h at 37 °C. A solution of 50 nM parathion was used as positive control. Afterwards, 200 μL of a reaction mixture containing 0.5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and 100 μM acetylthiocholine (ATCh) were added to each well. The resulting product of the reaction, 5-thio-2-nitrobenzoate (TNB), was measured at 410 nm every 90 s up to 60 min.

2.9. Morphology

The concentrations used for the morphological assay were the previously calculated EC₅₀ (24 h) values. These values were chosen as the highest exposure concentration along with the fractions EC₅₀/2 and EC₅₀/4. Undifferentiated and differentiated SH-SY5Y cells were exposed for 24 h. Afterwards, cells were directly fixed with a 1.6% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.2) for 60 min at 4 °C. Later, they all were postfixed in 1% osmium tetroxide during the same time and temperature. Time elapsed, samples were dehydrated in ethanol at progressively higher concentrations and em-

bedded in epoxy embedding medium (Epon). Toluidine blue-stained semi-thin sections (0.5 mm thick) used as controls were viewed in a Leit (Aristoplan) light microscope. Thin sections (60–80 nm thick) were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM-10 transmission electron microscope. Cell growth and development of morphology damage was observed using a Leica DMIL inverted microscope by phase contrast.
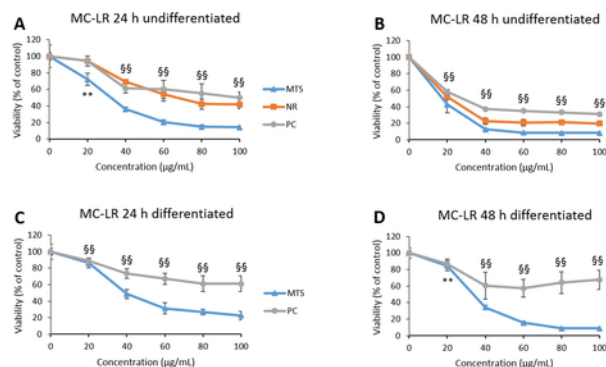
2.10. Calculations and statistical analysis

Data for the cytotoxicity assays and oxidative stress biomarkers were presented as mean±standard deviation (SD) in relation to control. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA). Differences were considered significant from $P < 0.05$. EC_{50} values were derived by linear regression in the concentration-response curves.

3. Results

3.1. Cytotoxicity assays

A concentration dependent decrease of both undifferentiated and differentiated SH-SY5Y cells viability was observed after their exposure to 1–100 µg/mL MC-LR at 24 and 48 h (Fig. 1). The EC_{50} values obtained in all the cytotoxicity assays performed are shown in Table

1. In the case of both differentiated and undifferentiated cells, MTS assay demonstrated to be the most sensitive biomarker, providing lower EC_{50} values in undifferentiated cells after the exposure times considered, compared to the differentiated cultures (Table 1).

Regarding to CYN, a concentration dependent decrease of viability was observed as well (Fig. 2). Using the MTS assay as a reference for its sensitivity, a higher cytotoxic response could be appreciated after 24 h of exposure in differentiated cells compared to the undifferentiated, that response is contrary to that obtained after 48 h of exposure (Table 1).

The concentration-response curves of the two cyanotoxins combination after the MTS assay, which demonstrated to be the most sensitive biomarker for both toxins in both types of cells, are shown in Fig. 3. The toxin combination proved to be more cytotoxic at the highest concentration tested on undifferentiated cells compared to the individual cyanotoxins after both exposure periods. On the contrary, on differentiated cells the response of the toxins combination was similar to the observed for MC-LR.

3.2. Assessment of the effect of cyanotoxins combination by the isobolograms method

In the experiment performed with undifferentiated cells, the cyanotoxin combination presented a CI > 1, which confirmed an antagonistic mode of action between these two toxins on SH-SY5Y cells (Table 2 and Fig. 4A and B). Data in Table 2 demonstrate that CI values were from antagonism (CI = 1.60–1.10) to moderate (CI = 1.45–1.08) effect over a wide range of EC_{50}-EC_{90} concentrations of MC-LR and CYN in combination. The strongest antagonistic effect was observed after 48 h. Moreover, this effect was more pronounced at lower concentrations.

In the experiment performed with the differentiated cells, the cyanotoxin combination presented a CI > 1, confirming again an antagonistic mode of action between these two toxins on differentiated SH-SY5Y cells (Table 3 and Fig. 4C and D). Data in Table 3 demonstrate that CI values were equivalent to an antagonistic effect (CI = 2.08–1.65) over the range of EC_{50}-EC_{90} concentrations of MC-LR and CYN in combination. The strongest antagonistic effect was observed after 24 h.

3.3. Oxidative stress assays

The exposure to 9.25, 18.5 or 37 µg/mL MC-LR leaded to no significant changes in the ROS assay in SH-SY5Y cells after 4, 8, 12 or 24 h of exposure. However, it showed significant differences after 24 h of exposure to all the concentrations tested in the GSH assay, and after 12 h of exposure to the highest concentration (Fig. 5).

Fig. 1. Reduction of tetrazolium salt (MTS), neutral red uptake (NR) and protein content (PC) on SH-SY5Y cells after 24 h (**A**) and 48 h (**B**) of exposure to 0–100 µg/mL MC-LR. All values are expressed as mean ± s.d. ** MTS significantly different from control group (p < 0.01), §§ all parameters significantly different from control group (p < 0.01).

Table 1
Cytotoxicity results of undifferentiated and differentiated SH-SY5Y cells exposed to MC-LR and CYN. Results are expressed as mean±s.d.

Toxin	Cell type	Time (h)	MTS assay		NR assay		PC assay	
			EC_{50} values (µg/mL)	Significant results (from) (µg/mL)	EC_{50} values (µg/mL)	Significant results (from) (µg/mL)	EC_{50} values (µg/mL)	Significant results (from) (µg/mL)
MC-LR	**Undifferentiated**	24	36.21±1.89	20	67.69±1.65	40	>100	40
		48	20.80±2.08	20	24.27±0.45	20	29.58±2.70	20
	Differentiated	24	44.30±0.91	20	–	–	>100	20
		48	37.01±1.71	20	–	–	>100	40
CYN	**Undifferentiated**	24	0.87±0.13	0.2	2.26±0.29	0.5	4.37±1.34	0.75
		48	0.32±0.08	0.2	1.27±0.27	0.4	2.01±0.29	0.4
	Differentiated	24	0.30±0.05	0.1	–	–	>10	2.5
		48	0.53±0.02	0.1	–	–	>10	1

Fig. 2. Reduction of tetrazolium salt (MTS), neutral red uptake (NR) and protein content (PC) on SH-SY5Y cells after 24 h (**A**) and 48 h (**B**) of exposure to 0–10 μg/mL CYN. All values are expressed as mean ± s.d. ** MTS significantly different from control group (p < 0.01), ¥ NR significantly different from control group (p < 0.05), §§ all parameters significantly different from control group (p < 0.01).
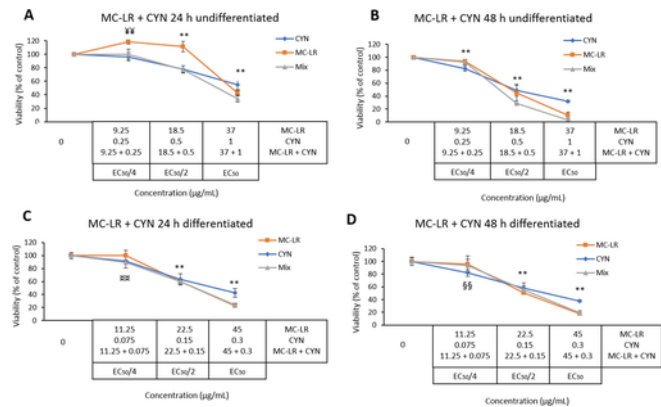
Fig. 3. Reduction of tetrazolium salt (MTS) on SH-SY5Y cells after 24 h (**A**) and 48 h (**B**) of exposure to different concentrations of MC-LR + CYN combinations at a ratio of CE_{50} MC-LR/CE_{50} CYN (37:1). All values are expressed as mean ± s.d. ** significantly different from control (p < 0.01) for the three toxins tested, and ¥¥ significantly different from control group (p < 0.01) for the MC-LR toxin alone.

After exposure to 0.25, 0.5 or 1 μg/mL CYN, no significant differences were observed in any of the exposure times assayed in either of the oxidative stress biomarkers evaluated (Fig. 6) compared to the control group.

Similarly to the cell behavior after toxins exposure individually, SH-SY5Y did not produced any significant difference in the ROS assay after the exposure to MC-LR+CYN combination, but it did after

4 h of exposure to the highest concentration, and after 8 h in the other two concentrations in the GSH assay (Fig. 7).

3.4. Acetylcholinesterase (AChE) activity

A significant increase of AChE activity on undifferentiated SH-SY5Y cells was observed only after the highest concentration of MC-LR assayed (37 μg/mL). However, neither CYN nor its combination with MC-LR induced significant changes in this enzymatic activity (Fig. 8A).

In the case of the differentiated cells, a significant decrease of AChE activity was shown after exposure to all CYN-concentrations assayed (0.075–0.3 μg/mL). Nevertheless, although MC-LR did not produce significant changes, exposure to the mixture at the highest concentrations (45 and 0.3 μg/mL of MC-LR and CYN, respectively) led to an increase in this enzymatic activity (Fig. 8B).

3.5. Morphology study

Unexposed undifferentiated SH-SY5Y cells observed under phase-contrast microscope showed scarce cytoplasmic projections, frequently two of them placed in opposite directions. These projections usually present connections with nearby cells (Fig. 9A). The light microscope revealed mitotic cells with big nuclei and nucleoli (Fig. 9B). Under electronic microscope, cells present irregular nuclei with heterochromatin condensation and big nucleoli. In the cytoplasm stand out a big number of free ribosomes, mitochondria were scarce and presented dense matrix (Fig. 9C). In the non-treated differentiated cells, a high number of cytoplasmic projections was observed under the phase-contrast microscope (Fig. 10A). Under light microscopy, cells presented a fusiform shape with endoplasmic reticulum dilatations and lipidic vacuoles (Fig. 10B). The electron microscope revealed a cell cytoplasm with a higher number of microtubules and intermediate filaments than the undifferentiated cells (Fig. 10C).

3.5.1. Microscope observations of cells exposed to pure MC-LR

Undifferentiated cells exposed to 37 μg/mL MC-LR presented, under phase-contrast, light and electronic microcopies, cell death signs, with a reduction of their size, chromatin condensation, and numerous vacuoles in the cytoplasm in fragmentation process. Moreover, apoptotic nuclei with chromatin and nucleolar segregation were observed (Fig. 11A–D). At the lowest concentrations (18.5 and 9.25 μg/ mL MC-LR), the most characteristic observations under phase-contrast microscope were the cytoplasmic projections by way of lamellipodium (Fig. 11E). The light microscope revealed cellular cycle arrest in the mitosis phase and nuclei with irregular shape and big nucleoli (Fig. 11F). Under the TEM, cells presented chromatin conden-

Table 2

The parameter m, D_m and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (IC_{50}), and the conformity of the data to the mass-action law, respectively. D_m and m values are used for calculating the CI value CI<1, indicates synergism (Syn); CI=1, indicates additive effect (Add); CI>1, indicates antagonism (Ant). IC_{50}, IC_{75} and IC_{90} are the doses required to inhibit proliferation 50, 75 and 90%, respectively. CalcuSyn software provide automatically the IC_{50}, IC_{75} and IC_{90} values.

Cyanotoxin	Time (hours)	D_m (μg/mL)	m	r	CI values (undifferentiated cells)		
					CI_{50}	CI_{75}	CI_{90}
MC-LR	24	44.02	5.20	0.86603			
	48	19.13	3.51	0.99320			
CYN	24	1.01	2.20	0.98604			
	48	0.58	1.66	0.97832			
MC-LR + CYN	24	29.30	5.44	0.96064	1.45 Ant	1.24 Ant	1.08 Add
	48	16.17	4.25	0.99509	1.60 Ant	1.31 Ant	1.09 Add

Fig. 4. Combination index (CI)/fraction affected (fa) curve in undifferentiated (**A, B**) and differentiated (**C, D**) SH-SY5Y cells exposed to a MC-LR and CYN binary combination after 24 h and 48 h of exposure. Each point represents the CI±s.d. at a fractional effect. The dotted line (CI=1) indicates additivity, the area under the dotted line points out a synergist effect, and the area above the dotted line signify antagonism.

sation in the nuclear membrane, segregated nucleoli and formation of autophagosomes (Fig. 11G and H).

Differentiated SH-SY5Y cells exposed to 45 μg/mL MC-LR showed an elevated refringence under the phase-contrast microscope caused by numerous dead cells and apoptotic bodies, as it could also be observed by light microscopy (Fig. 12A and B). Under electron microscopy, the presence of a large quantity of confluent heterophagosomes, nucleolar segregation, and numerous lipidic vacuoles were observed (Fig. 12C and D). At the lowest concentrations assayed (22.5 and 11.25 μg/mL MC-LR), cells observed under phase-contrast, light and electronic microcopies presented protein granules, possibly caused by alterations in the protein folding, lipidic vacuoles and chromatin condensation. Mitotic processes were also observed, although this phenomenon could be stopped due to the adverse situation induced by MC-LR (Fig. 12E–J).

3.5.2. Microscope observations of cells exposed to pure CYN

At the highest concentration assayed (1 μg/mL), undifferentiated cell cultures presented clear morphological alterations leading to cell death such as apoptotic bodies, and heterochromatin condensation (Fig. 13A and B). Ultrastructurally, nuclei presented irregular shape and, frequently, the presence of apoptotic nuclei is high, with cytoplasmic fragmentation. In addition, segregated nucleolus was observed, being only visible their fibrillar component (Fig. 13C). At 0.5 and 0.25 μg/mL CYN, no remarkable morphological alterations were observed under light microscopy, where it is possible to observe the cellular cycle in mitosis phase. The TEM showed euchromatic nuclei and numerous mitochondria in the cytoplasm, a characteristic of undifferentiated cells (Fig. 13D and E).

After differentiation, SH-SY5Y cells exposed to the highest CYN concentration (0.3 μg/mL CYN) experienced a massive cell death, easily observable by phase-contrast (high refraction) and light (many cellular debris) microscopies. No cell division was observed (Fig. 14A and B). Ultrastructurally, apoptotic nuclei with heterochromatin condensation were appreciated, together with the presence of heterophagosomes (Fig. 14C). After exposure to 0.15 and 0.075 μg/mL CYN, cells presented an increased number of ribosomes compared to control cells. Moreover, an increment in lipidic vacuoles and dilated endoplasmic reticuli was observed. Nuclei presented the typical apoptotic morphology (Fig. 14D–I).
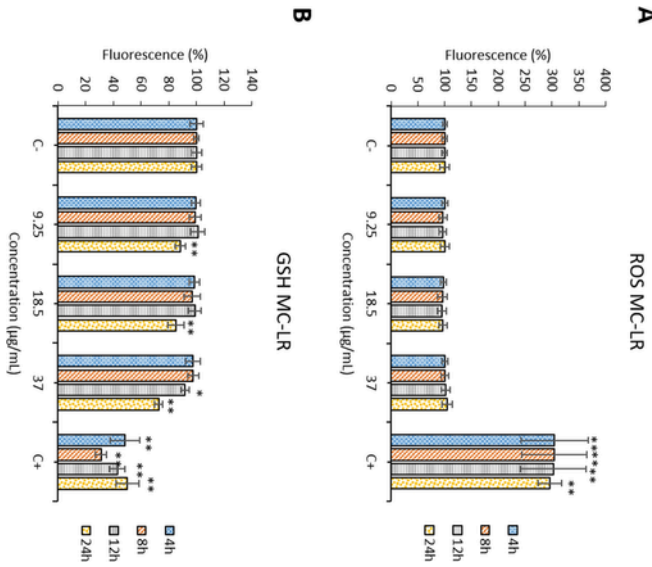
3.5.3. Microscope observations of cells exposed to the combination MC-LR/CYN

Concerning the combinations, the combination with the highest CYN and MC-LR concentrations (1 + 37 μg/mL) induced and intense cell death in undifferentiated cells, mainly by apoptosis, appearing numerous cellular debris and apoptotic bodies. Moreover, apoptotic nuclei with chromatin condensation were observed (Fig. 15A and B). The combination composed by 0.5 + 18.5 μg/mL induced a moderate cell death by apoptosis, were cells presented blisters in the surface, a typical observation in cells dying by apoptosis (Fig. 15C and D).

Differentiated cells presented two extremes. At the highest concentration assayed (0.3 + 45 μg/mL CYN + MC-LR), a remarkable cell death was observed, with the presence of pre-apoptotic bodies all over the culture. On the other hand, when cells were exposed to the other two concentrations, zones without any visible damage and zones with intense cellular death were perceived (Fig. 16A–C). The TEM showed numerous indicators of cellular damage in cells exposed to the highest exposure concentration. Thus, lipidic degeneration, apoptotic nuclei, endoplasmic reticulum with protein concentration, pre-apoptotic bodies, heterophagosomes, nuclear bodies, and nucleolar segregation were observed (Fig. 16D–G).

4. Discussion

Microcystin-LR and CYN have been extensively studied *in vitro* in hepatic and renal cell lines (Chen and Xie, 2016; McLellan and Manderville, 2017; Pichardo et al., 2017). However, the studies concerning neuronal cell lines are still scarce, although some *in vivo* studies point out that these cyanotoxins could induce neurotoxic effects (Guzmán-Guillén et al., 2015; Kist et al., 2012; Qian et al., 2018; Wu et al., 2016, 2017). Neurotoxicity of cyanotoxins has been reviewed, including the main mechanisms and effects (Florczyk et al., 2014; Hu et al., 2016) although the molecular mechanisms underlying these effects have not been still elucidated yet. In this sense, the present work focused on the potential effects induced by MC-LR, CYN, and their combination in the human neuronal SH-SY5Y cell line. For MC-LR, only Zhang et al. (2018) used this experimental

Table 3
The parameter m, D_m and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (IC_{50}), and the conformity of the data to the mass-action law, respectively. D_m and m values are used for calculating the CI value CI<1, indicates synergism (Syn); CI=1, indicates additive effect (Add); CI>1, indicates antagonism (Ant). IC_{50}, IC_{75} and IC_{90} are the doses required to inhibit proliferation 50, 75 and 90%, respectively. CalcuSyn software provide automatically the IC_{50}, IC_{75} and IC_{90} values.

Cyanotoxin	Time (hours)	D_m (μg/mL)	m	r	CI values (differentiated cells)		
					d	CI_{75}	CI_{90}
MC-LR	24	30.17	4.14	0.96974			
	48	28.09	4.06	0.97042			
CYN	24	0.25	2.32	0.96974			
	48	0.23	2.53	0.96292			
MC-LR + CYN	24	27.32	2.37	0.99939	1.65 Ant	1.84 Ant	2.08 Ant
	48	26.50	3.00	0.99411	1.72 Ant	1.77 Ant	1.83 Ant

Fig. 5. Reactive oxygen species (ROS) and reduced glutathione (GSH) levels on SH-SY5Y cells after 4, 8, 12 and 24 h of exposure to 0–37 μg/mL MC-LR. Cells exposed to medium without serum were used as negative control in both assays (C-). Cells exposed to 200 μM MnCl$_2$·4H$_2$O and exposed to 1 μM BSO were used as positive control (C+) in the case of the ROS and the GSH assays, respectively. All values are expressed as mean ± s.d. The significance levels observed are * p < 0.05 and **p < 0.01 significantly different from control group.
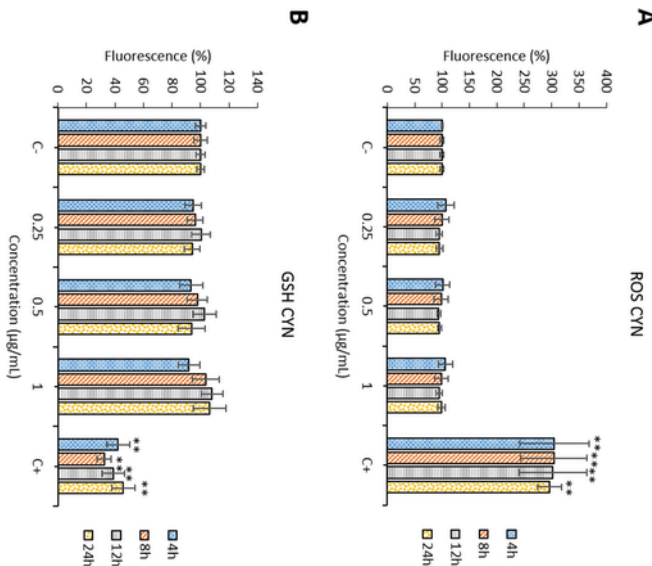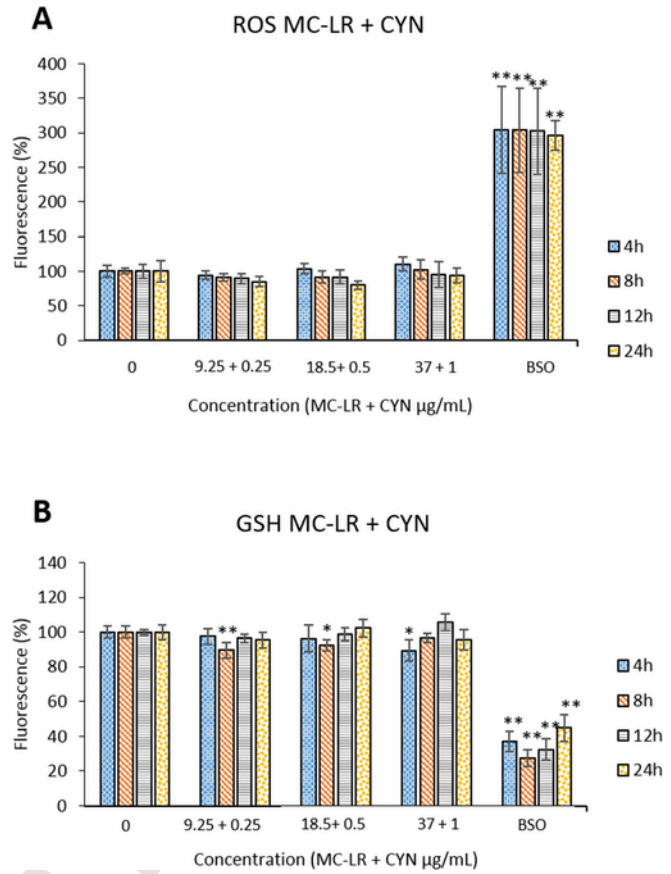

Fig. 6. Reactive oxygen species (ROS) and reduced glutathione (GSH) levels on SH-SY5Y cells after 4, 8, 12 and 24 h of exposure to 0–1 μg/mL CYN. Cells exposed to medium without serum were used as negative control in both assays (C-). Cells exposed to 200 μM MnCl$_2$·4H$_2$O and exposed to 1 μM BSO were used as positive control (C+) in the case of the ROS and the GSH assays, respectively. All values are expressed as mean ± s.d. ** Significantly different from control group (p < 0.01).

model but for different purposes, such as transport, bioaccumulation, hyperphosphorylation of PP2A-dependent Tau sites, and cell death. In the case of CYN and its combination with MC-LR, the present study shows their effects *in vitro*, contributing to mend the lack of information about this matter.

Regarding to MC-LR, our results showed a decrease of the cell viability after 24 and 48 h of exposure to MC-LR in both undifferentiated and differentiated cells, being the undifferentiated the most sen-

Fig. 7. Reactive oxygen species (ROS) and reduced glutathione (GSH) levels on SH-SY5Y cells after 4, 8, 12 and 24 h of exposure to different concentrations of MC-LR + CYN combinations at a ratio of CE$_{50}$ MC-LR/CE$_{50}$ CYN (37:1). Cells exposed to medium without serum were used as negative control in both assays (C-). Cells exposed to 200 μM MnCl$_2$·4H$_2$O and exposed to 1 μM BSO were used as positive control (C+) in the case of the ROS and the GSH assays, respectively. All values are expressed as mean ± s.d. The significance levels observed are * p < 0.05 and **p < 0.01 significantly different from control group.

sitive cells. According to our results, several authors have described a reduction of cell viability in different neuronal cell lines exposed to MC-LR (primary murine WBC, primary murine CGNs cells, primary hippocampal neurons, RAW246.7 murine macrophage-like cells, BV-2 cells, N2a cells, GT1-7 cells and SH-SY5Y cells) (Cai et al., 2015; Ding et al., 2017; Feurstein et al., 2009, 2011; Li et al., 2015; Takser et al., 2016; Zhang et al., 2018). However, most of these authors used MC-LR concentrations up to 10 μM for 24, 48 or 72 h of exposure, and although they described decreases in cell viability, only Cai et al. (2015) referred an EC$_{50}$ value for pure MC-LR. These authors exposed cells up to 30 μM MC-LR, establishing an EC$_{50}$ of 10 μM MC-LR in primary hippocampal neurons after 24 h of exposure using the MTT cytotoxicity assay. In this regard, the presence of organic anion transporting polypeptide transporters (OATPs) has been described as an important requirement to MC-LR toxicity. It is well known that the OATP1B subfamily members are MCs transporters (Fischer et al., 2005). Fischer et al. (2005) suggested that OATP1A2 transporters expressed in brain capillary endothelial cells and in the cell membrane of human neurons could be involved in MC-LR transport through the blood-brain barrier. In addition, Ding et al. (2017) demonstrated the role of the Oatp1a5 transporting MC-LR into neuronal cells. These facts support that MC-LR damage could only evolve if it is transported into the nervous system through OATPs or other different
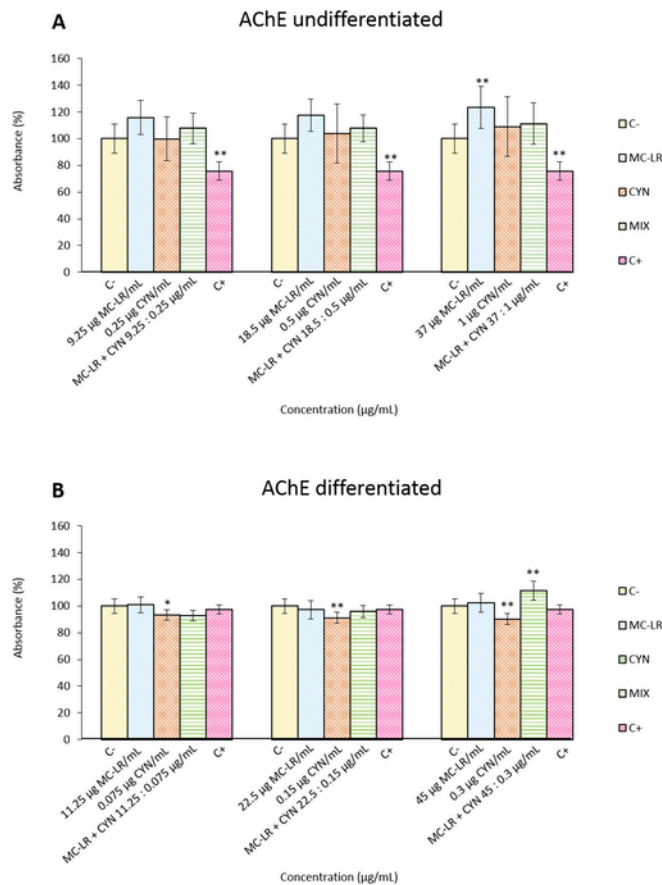
Fig. 8. Acetylcholinesterase activity (AChE) on undifferentiated and differentiated SH-SY5Y cells exposed to MC-LR (0–37 or 0–45 μg/mL, respectively), CYN (0–1 or 0.3 μg/mL, respectively) or MC-LR + CYN combination after 24 h. Cells exposed to medium without serum were used as negative control in both assays (C-). Cells exposed to 50 nM parathion were used as positive control (C+). All values are expressed as mean ± s.d. ** Indicates significant difference from control group value (p < 0.01).
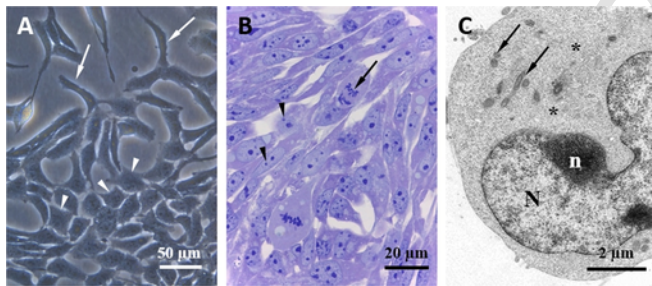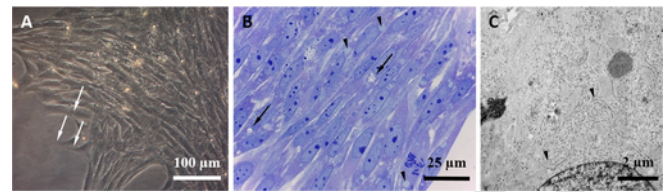

Fig. 9. Morphology of control undifferentiated SH-SY5Y cells after 24 h of exposure to nutrient medium without serum. Contrast-phase microscopy of a SH-SY5Y cell culture in normal neuronal growth. Cells present cytoplasmic projections contacting with other cells (arrows) and the morphological characteristics of an epithelial culture (arrowheads). Bar = 50 μm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells in mitosis processes (arrows) with big nucleoli in the nucleus (arrowheads). Bar = 25 μm (**B**). Transmission electronic microscopy of SH-SY5Y cells with euchromatic nuclei (N) and dense nucleoli (n). Free ribosomes (asterisk) and scarce mitochondria (arrows). Bar = 2 μm (**C**).

transporters (Feurstein et al., 2009, 2011). However, the presence of these transporters in the nervous system is scarce and, in some cases, their number can vary with the differentiation process (Rozman et al., 2017; Yagdiran et al., 2016). Thus, the low MC-LR-toxicity in those cell lines (primary murine WBC, primary murine CGNs cells, pri-
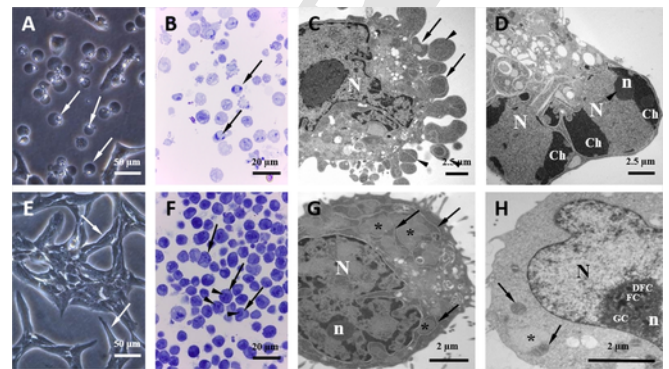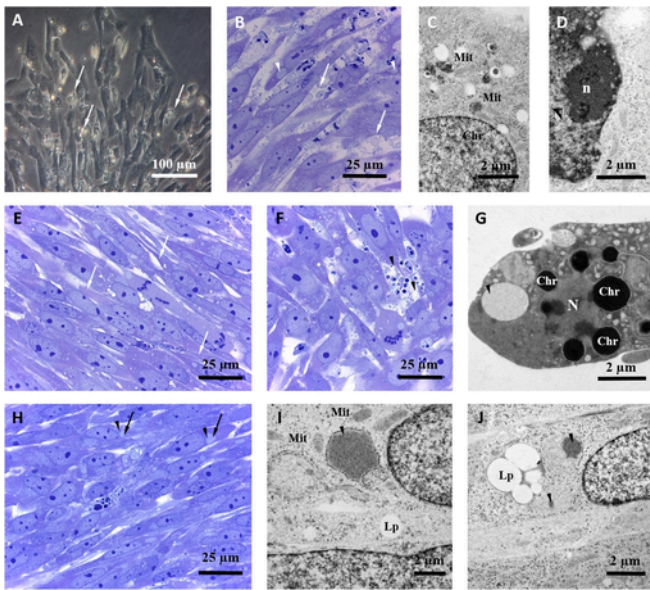

Fig. 10. Morphology of control differentiated SH-SY5Y cells after 24 h of exposure to nutrient medium without serum. Contrast-phase microscopy of a SH-SY5Y cell culture in normal neuronal growth. Cells present cytoplasmic projections contacting with other cells (arrows). Bar = 100 μm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells in mitosis processes (asterisk). Endoplasmic reticulum dilatations (arrowheads) and lipidic vacuoles (arrows). Bar = 25 μm (**B**). Transmission electronic microscopy of SH-SY5Y cells presenting numerous microtubules and intermediate filaments (arrowheads). Bar = 2 μm (**C**).

Fig. 11. Morphology of undifferentiated SH-SY5Y cells after 24 h of exposure to MC-LR. Phase-contrast microscopy of cells exposed to 37 μg/mL MC-LR. Rounded cells (arrows) with clear signs of cell death. Bar = 50 μm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 37 μg/mL MC-LR. Nuclei with condensed chromatin (arrows) as clear sign of cell death. Bar = 20 μm (**B**). Transmission electronic microscopy of SH-SY5Y cells exposed to 37 μg/mL MC-LR. Irregular nuclei (N), pseudopods retraction, appearance of blisters in the cellular membrane (arrows), and presence of apoptotic bodies (arrowheads). Chromatin condensation (Ch) and nucleolar segregation of apoptotic nuclei (N). Nucleolus (n). Bar = 2.5 μm (**C, D**). Phase-contrast microscopy of cells exposed to 9.25 μg/mL MC-LR. Enlarged cells with cytoplasmic elongations as lamellipodiums (arrows). Bar = 50 μm (**E**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 18.5 μg/mL MC-LR. Cells with irregular shape (arrows) presenting big nucleoli (arrowheads). Bar = 20 μm (**F**). Transmission electronic microscopy of SH-SY5Y cells exposed to 18.5 and 9.25 μg/mL MC-LR. Altered mitochondria rounded by endoplasmic reticulum cisternae (arrows) forming autophagosomes (asterisk). Euchromatic nucleus (N) with a big nucleolus (n) where it is possible to observe the granulas component (GC), the dense fibrillary component (DFC) and the fibrillary center (FC). Bar = 2 μm (**G, H**).

mary hippocampal neurons, RAW246.7 murine macrophage-like cells, BV-2 cells, N2a cells, GT1-7 cells and SH-SY5Y cells) and the increment in the EC_{50} value in the differentiated SH-SY5Y cells from our study, compared with the undifferentiated ones, could be due to this fact.

The potential mechanisms by which MC-LR induces its neurotoxic effects include effects on neurotransmitters, neurochannels, signal transduction, oxidative stress and cytoskeleton disruption (Hu et al., 2016). The neurotoxicity of MCs seems a multi-pathway process, although the molecular mechanisms remain evasive. In this sense, Cai et al. (2015) described that MC-LR disrupt calcium homeostasis in neurons, inducing a concentration-dependent increment of intracellular free Ca^{2+} levels from stores together with a decrease in cell viability. In agreement with these findings, Li et al. (2015) reported that increased intracellular Ca^{2+} levels led to an activation of the phosphatase calcineurin, which result in apoptosis via dephosphorylation of the proapoptotic Bcl-2 family member Bad. This calcium release, together with the associated cytochrome C release, also activates the

Fig. 12. Morphology of differentiated SH-SY5Y cells after 24 h of exposure to MC-LR. Phase-contrast microscopy of cells exposed to 45 μg/mL MC-LR. Rounded cells (arrows) with clear signs of cell death. Bar = 100 μm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 45 μg/mL MC-LR. Presence of numerous apoptotic bodies (arrows) as clear sign of cell death. Presence of dilated endoplasmic reticulum (arrowheads). Bar = 25 μm (**B**). Transmission electronic microscopy of differentiated SH-SY5Y cells exposed to 45 μg/mL MC-LR. Chromatin condensation (Chr), presence of numerous mitochondria (Mit), and nucleolar segregation of apoptotic nuclei (N). Nucleolus (n).Bar = 2.5 μm (**C, D**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 22.5 μg/mL MC-LR. Enlarged cells with cytoplasmic elongations as lamellipodiums (arrows) and presence of apoptotic cellular debris (arrowheads). Bar = 25 μm (**E, F**). Transmission electronic microscopy of differentiated SH-SY5Y cells exposed to 22.5 μg/mL MC-LR. Cells with irregular shape presenting nuclei (N) with chromatin condensation (Chr). Bar = 2 μm (**G**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 11.25 μg/mL MC-LR. Numerous dilated endoplasmic reticuli (arrows) with protein condensation (arowheads). Bar = 25 μm (**H**). Transmission electronic microscopy of differentiated SH-SY5Y cells exposed to 11.25 μg/mL MC-LR. Presence of mitochondria (Mit), protein condensation (arrowheads) and lipidic vacuoles (Lp). Bar = 2 μm (**I, J**).

caspases protein family, well-known apoptotic proteins. Thus, Feurstein et al. (2011) and Rozman et al. (2017) demonstrated that MC-LR induced cell death by apoptosis through the activation of caspase proteins in primary murine CGN cells and primary rat astrocytes, respectively. These findings support our results, since our most sensitive cytotoxicity biomarker was the MTS assay, which assesses the mitochondrial health and its activity, which is related with cell death (Tait and Green, 2013). Zhang et al. (2018) demonstrated that 10 μM MC-LR with endoporter caused cell death in SH-SY5Y cells by using the lactate dehydrogenase (LDH) release assay. In this experimental model, the authors indicated that MC-LR induced phosphorylation of protein Tau, promoting dissociation of Tau from microtubules and aggregation of phospho-paired helical filaments-Tau, and consequently, neuronal degeneration and cell death. Cytoskeleton disruption is considered to be one of the cytotoxicity triggering caused by MC-LR (Hu et al., 2016) based on the alterations induced *in vivo* by the toxin in diverse cytosqueletal proteins in brains of rats (Zhao et al., 2015), and in *vitro* experiments (Meng et al., 2011).

Cellular death can be also corroborated through morphological studies. In this regard, the present work shows how MC-LR induced the most common characteristics of cellular death such as cytoplasm fragmentation, chromatin condensation, and nucleolar segregation, endoplasmic reticulum dilatation, lipidic vacuoles, and presence of heterophagosomes, both in the undifferentiated and differentiated
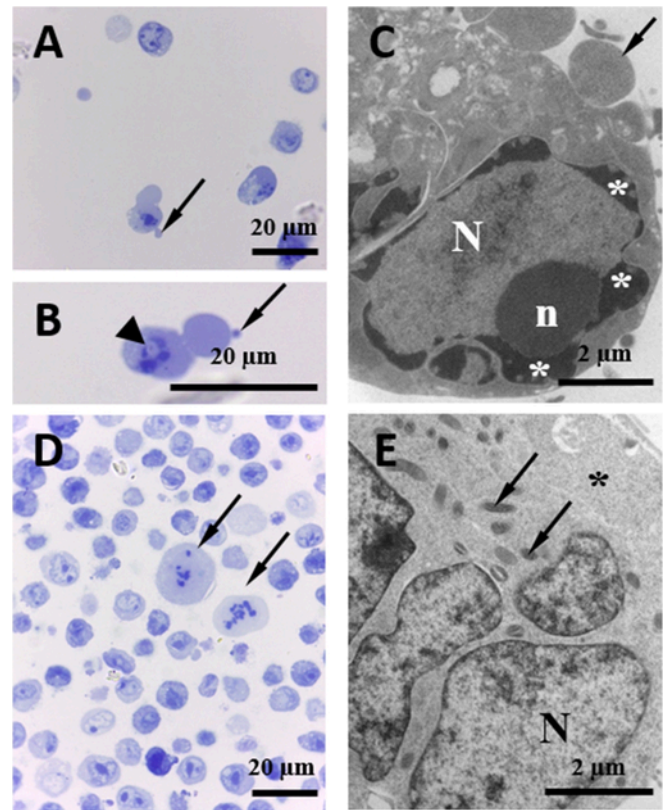
Fig. 13. Morphology of undifferentiated SH-SY5Y cells after 24 h of exposure to CYN. Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 1 μg/mL CYN. Detention of cellular growth and decrease of cell number caused by cellular death by apoptosis. Apoptotic bodies (arrows). Heterochromatin in apoptotic nucleus (arrowhead). Bar = 20 μm (**A, B**). Transmission electronic microscopy of SH-SY5Y cells exposed to 1 μg/mL CYN. Fractionated cytoplasm and formation of apoptotic bodies (arrows). Condensed chromatin in the inner face of the nucleolar membrane (asterisks). Fibrillar component of the nucleolus (n). Nucleus (N). Bar = 2 μm (**C**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 0.5 μg/mL CYN. No evidence of morphological alterations. Bar = 20 μm (**D**). Transmission electronic microscopy of SH-SY5Y cells exposed to 0.5 and 0.25 μg/mL CYN. Euchromatic nuclei with irregular shape (N). Cytoplasm with numerous free ribosomes (asterisks). Mitochondria (arrows). Bar = 2 μm (**E**).

SH-SY5Y cells. In this sense, Feurstein et al. (2011) found that MC-LR induced a slight impairment of the neurite network in primary murine CGN cells. In addition, Meng et al. (2011) described some apoptotic effects such as the reorganization of cytoskeletal architectures in differentiated PC12 cells exposed to 10 μM MC-LR, which was also observable in the differentiated SH-SY5Y cells from our study. Moreover, Zhang et al. (2018) described neurites degeneration and cell death in SH-SY5Y cells exposed to 10 μM MC-LR.

Two other toxic mechanisms studied in the present work are the oxidative stress generation and the AChE disruption. For the oxidative stress evaluation two related parameters were studied, ROS generation and GSH depletion, since it has been stated that free-radical damage is one of the toxic mechanisms of MC-LR (Meng et al., 2013). In the present work, ROS levels did not suffer any alteration in undifferentiated cells exposed to pure MC-LR. However, Meng et al. (2013) found that concentrations up to 10 μM MC-LR induced significant enhancement of ROS levels at early times, reaching the highest levels at 3 h after the exposure. Nonetheless, these authors also found that after this time, ROS levels started to decline to basal levels due to a rapid response of cells (differentiated PC12 cells) against MC-LR. Moreover, in the present study, GSH levels decreased after 24 h of exposure at all the concentrations assayed. This could confirm

Fig. 14. Morphology of differentiated SH-SY5Y cells after 24 h of exposure to CYN. Phase-contrast microscopy of cells exposed to 0.3 μg/mL CYN. Rounded cells (arrows) with clear signs of cell death. Bar = 100 μm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 0.3 μg/mL CYN. Presence of numerous apoptotic bodies (arrows) as clear sign of cell death. Presence of dilated endoplasmic reticulum (arrowheads). Bar = 25 μm (**B**). Transmission electronic microscopy of SH-SY5Y cells exposed to 0.3 μg/mL CYN. Condensed chromatin in the inner face of the nucleolar membrane (Chr). Bar = 2 μm (**C**). Phase-contrast microscopy of cells exposed to 0.15 μg/mL CYN. Rounded cells (arrows) with clear signs of cell death. Bar = 100 μm (**D**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 0.15 μg/mL CYN. Presence of dilated endoplasmic reticulum (arrowheads) and lipidic vacuoles (arrows). Bar = 25 μm (**E**). Transmission electronic microscopy of SH-SY5Y cells exposed to 0.15 μg/mL CYN. Condensed chromatin (Chr) in apoptotic nucleus (N). Bar = 2 μm (**F**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 0.075 μg/mL CYN. Presence of numerous apoptotic bodies (arrows) as clear sign of cell death. Bar = 25 μm (**G**). Transmission electronic microscopy of SH-SY5Y cells exposed to 0.075 μg/mL CYN. Euchromatic nuclei with irregular shape (N). Dilate endoplasmic reticulum (ER) and lipidic vacuoles (Lp). Bar = 2 μm (**H**).

the fact that ROS levels appeared unaffected since GSH could directly against ROS (Circu and Aw, 2010). These oxidative stress parameters were not evaluated in differentiated SH-SY5Y cells since several authors have highlighted that these cells are more resistant to oxidative stressors due to changes in mitochondrial metabolism and antioxidant defenses (Cecchi et al., 2008; Cheung et al., 2009; Schneider et al., 2011). In contrast, diverse *in vivo* studies have reported the implication of oxidative stress in the neurotoxicity induced by MC-LR (Li et al., 2014 Mello et al., 2018; Wang et al., 2010; Zhao et al., 2015).

Concerning the AChE activity, this enzyme is a well-known biomarker of neuronal damage, as the affectation of the cholinergic system could lead to a malfunctioning of the locomotor system, behavior and cognitive processes (Kist et al., 2012). MCs may influence brain AChE indirectly via the inhibition of serine/threonine phosphatases (Hu et al., 2016). In our study, a significant increase of AChE activity was observed when undifferentiated SH-SY5Y cells were exposed to the highest concentration of MC-LR (37 μg/mL). The increased AChE activity could lead to a reduction of the cholinergic neurotransmission efficiency because of the lack of acetylcholine in the synaptic space, possibly contributing to a progressive cognitive impairment (Teodorak et al., 2015). Despite this, some other authors have demonstrated that AChE plays a role by promoting or suppressing cell death. An enhanced AChE activity take part in apoptosis, participating in the formation of apoptosomes or influencing the expression of
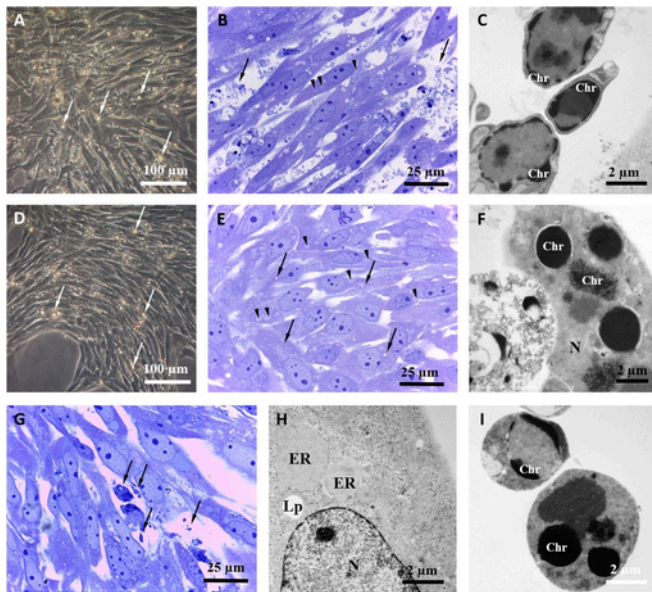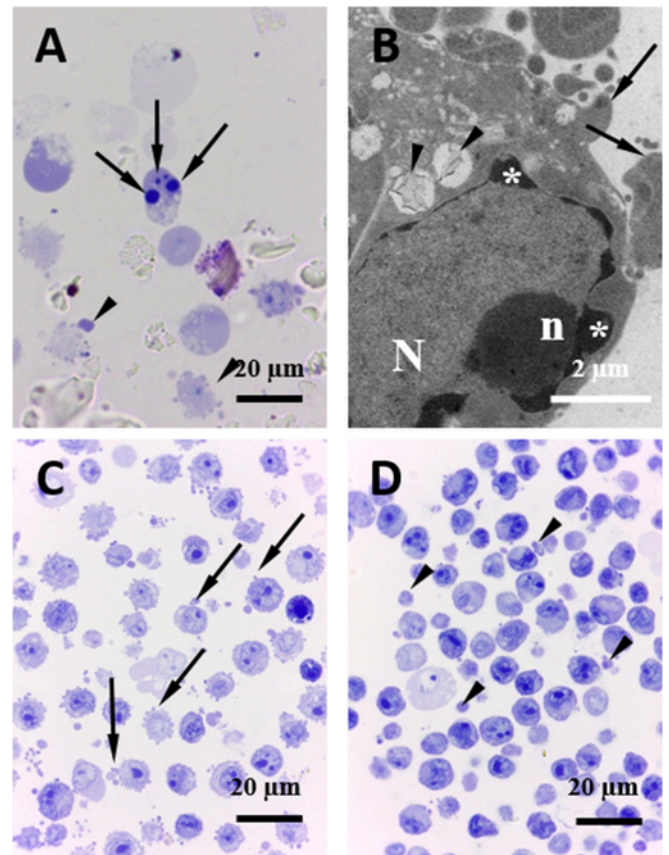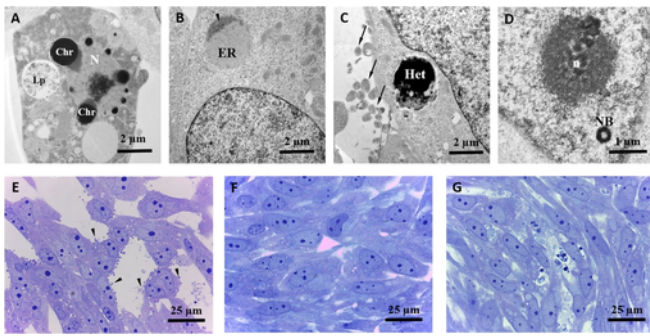

Fig. 15. Morphology of undifferentiated SH-SY5Y cells after 24 h of exposure to MC-LR + CYN. Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 37 μg/mL MC-LR + 1 μg/mL CYN. Intense cellular death by apoptosis. Heterochromatin accumulations in apoptotic nuclei (arrows). Cellular debris as apoptotic bodies (arrowheads). Bar = 20 μm (**A**). Transmission electronic microscopy of SH-SY5Y cells exposed to 37 μg/mL MC-LR + 1 μg/mL CYN. Heterochromatin condensation in the inner face of the nuclear membrane (asterisks). Nucleolar segregation with the fibrillary component (n). Apoptotic bodies formation (arrows). Autophagy vacuoles in the cytoplasm (arrowheads). Nucleus (N). Bar = 2 μm (**B**). Semithin sections of SH-SY5Y cells stained with toluidine blue. Cells exposed to 18.5 μg/mL MC-LR + 0.5 μg/mL CYN. Blister formation in cellular surface (arrows). Apoptotic bodies (arrowheads). Bar = 20 μm (**C, D**).

apoptotic genes (Park et al., 2004; Ben-Ari et al., 2006; Zhu et al., 2007). Zhu et al. (2007) also stated that AChE expression during apoptosis is associated with calcium mobilization. These facts are in agreement with our findings, since it has been shown that MC-LR induced cell death by apoptosis at the highest concentration assayed, correlating this effect with the AChE activity enhancement. On the contrary, when differentiated SH-SY5Y cells were exposed to 45 μg/mL MC-LR, no significant changes in the AChE activity were detected compared to the control group. This could be due to structural and functional modifications of the cells after the differentiation process, which could impede the effects of MC-LR over the AChE activity. However, cell death by apoptosis was also observed in these cells, which implies that not only the rise of the AChE could be involved in the cellular death, but also other potential factors such as intracellular calcium levels etc., that should be further investigated. Other authors have also evaluated the AChE activity disruption induced by MC-LR, but their studies have been carried out in *vivo* systems (Kist et al., 2012; Qian et al., 2018; Wu et al., 2016, 2017), obtaining contradictory and not conclusive results, depending on the administration route, or the experimental model assayed, etc. Conse-

Fig. 16. Morphology of differentiated SH-SY5Y cells after 24 h of exposure to MC-LR + CYN. Transmission electronic microscopy of SH-SY5Y cells exposed to 45 μg/mL MC-LR + 0.3 μg/mL CYN. Apoptotic nucleus (N) with heterochromatin condensation (Chr). Lipidic degeneration (Lp). Dilated endoplasmic reticulum (ER) with protein condensation (arrowheads). Pre-apoptotic bodies (arrows). Heterophagosome (Het). Nucleolus (n) with the presence of a nuclear body (NB). Bar = 2 μm (**A, B, C, D**). Semithin sections of cells culture were stained with toluidine blue. Cells exposed to 45 μg/mL MC-LR + 0.3 μg/mL CYN. Clear formation of apoptotic bodies (arrowheads). Bar = 25 μm (**E**). Cells exposed to 22.5 μg/mL MC-LR + 0.15 μg/mL CYN and 11.25 μg/mL MC-LR + 0.075 μg/mL CYN. No significant alterations observed. Bar = 25 μm (**F, G**).

quently, further research should be carried out in order to clarify the effects of MCs on this key enzyme in the nervous system.

Concerning CYN, a very scarce number of studies have been carried out in order to elucidate its neurotoxicity. However, due to its zwitterionic behavior and its small size, CYN is likely to be taken by the cells through diffusion, being able the crossing through the blood brain barrier (Florczyk et al., 2014; Valério et al., 2010). In the present study, CYN showed EC_{50} values even lower than MC-LR both in undifferentiated and differentiated cells. In agreement, Takser et al. (2016) found that pure CYN reached an EC_{50} value between 0.1 and 10 μM CYN in N2a cells after 24, 48 and 72 h of exposure. These authors also found that CYN induced almost a total cellular death at a concentration of 10 μM in RAW264.7 and BV-2 cells. These findings are also in agreement with the morphological results, which showed clear signs of cellular death by apoptosis in both types of SH-SY5Y cells exposed to 1 μg/mL (2.4 μM) and 0.3 μg/mL (0.7 μM) CYN. Relative to oxidative stress, although numerous works demonstrate *in vitro* the oxidative stress induction by CYN in different cell lines (reviewed by Pichardo et al., 2017), this work investigated the potential effects on ROS and GSH levels in the undifferentiated SH-SY5Y cell line. However, no effects were found, which is in disagreement with some *in vivo* studies, where oxidative stress was a main component of the cellular damaged observed (Guzmán-Guillén et al., 2015; Da Silva et al., 2018). These discrepancies could be due to the different experimental model or the concentrations used.

In relation to the AChE activity disruption, to our knowledge, no other papers have been published concerning the effects of CYN on AChE activity *in vitro*. In the present work, our results did not indicate any alteration at all CYN concentrations assayed (up to 1 μg/mL) in undifferentiated cells. This is in agreement with Da Silva et al. (2018), who described no significant differences in the AChE activity in brain of fish (*Hoplias malabaricus*) exposed to pure CYN after 7- and 14-days post treatment. In addition, these authors also reported increased AChE activity by 44% in brain at 7 days of exposure to aqueous CYN-producing cyanobacteria extracts, returning to control levels after 14 days. However, when differentiated SH-SY5Y cells were exposed to 0–0.3 μg/mL CYN, cultures showed a significant decrease of AChE activity at all CYN concentrations. These observations are in agreement with the findings reported by Guzmán-Guillén et al. (2015), who found a significant inhibition of 35% of AChE ac-

tivity in the brain of tilapia subchronically exposed to CYN (10 μg/L CYN) by immersion in an *A. ovalisporum* culture for 14 days has been reported. In this study, after a depuration process (7 days) a recovery of the enzyme was found. These contrary responses could be due to differences in the experimental conditions, highlighting the need to perform further studies.

Once the neurotoxic effects of pure MC-LR and CYN were studied separately, it was interesting to study their effects in combination, since these toxins can appear together in the nature (Bittencourt-Oliveira et al., 2014; Oehrle et al., 2010; Vasas et al., 2004). In this sense, in the present work the MC-LR + CYN combination resulted more cytotoxic than each individual toxin after 24 and 48 h of exposure in undifferentiated SH-SY5Y cells. However, the differentiation process resulted in a lower cytotoxicity of the toxin combination, as the effect of MC-LR + CYN was similar to that obtained for MC-LR. The combination interaction was analyzed by the isobolograms method described by Chou and Talalay (1984), which establish the foundations for assessing whether cytotoxicity induced by a combination of cyanotoxins is more or less harmful than the expected for individual cyanotoxins. This method is independent of the mode of action of the compounds and considers both the potency (EC_{50}, Dm) and the shape (m) of the dose-effect curve for each toxin (Ruiz et al., 2011a, 2011b). The method allows a prediction of synergism/antagonism at all effect levels (*fa*) for a combination of a different number of cyanotoxins. Depending on the equipotency level of MC-LR and CYN, it is feasible the cyanotoxin interaction can vary between antagonism and additivity. To explore this, the IC values at 50% inhibition (IC_{50}) and 90% inhibition (IC_{90}) were determined. The combined effect of the combination observed on undifferentiated SH-SY5Y cells is of antagonistic nature, with a slightly tendency to additivity at higher concentrations. In differentiated cells, only an antagonistic behavior was observed. However, experimental cytotoxicity and histopathological changes obtained by the combination appeared to be more related with additivity than antagonism in both cell types, although the antagonistic effect seems to be probable since the effect of the combination was not as intense as it could be expected. Similar results were obtained by Gutiérrez-Praena et al. (2018) in the hepatic cell line HepG2, where the combination of both cyanotoxins also presented an antagonistic response in the cells. This response could be due to complex dissimilar actions of these different cyanotoxins, although the mechanisms of interaction remain unknown. However, it is difficult to give an explanation to this phenomenon because of the isobolograms method only allows quantitative determination of synergism or antagonism, and the elucidation of the mechanism by which these relations occur is a separate issue that requires a different kind of approach (Lu et al., 2013). Similar to our results, Takser et al. (2016) found that a combination of MC-LR, CYN and anatoxin-a in an equimolar proportion (3.33 μM) induced a significant reduction of cell viability in N2a cells after 24, 48 and 72 h of exposure. Moreover, these authors also found that this combination induced a total cell death in the RAW264.7 and BV-2 cell lines. They also suggested that the combination was more toxic compared with the individual compounds. Regarding cell death, Takser et al. (2016) showed that the combination induced clear signs of cell apoptosis in all the cell lines used. This is in agreement with our morphological results, where an intense cell death was observed at the highest concentrations of the combination assayed for both SH-SY5Y cell types (37 μg/mL MC-LR + 1 μg/mL CYN and 45 μg/mL MC-LR + 0.3 μg/mL CYN for undifferentiated and differentiated cultures, respectively), mainly by apoptosis. Concerning oxidative stress and the AChE activity, no significant changes were observed respect to the control group at any of the combination concentrations assayed in the

undifferentiated cells. However, in the differentiated SH-SY5Y cells a significant increase of AChE activity was observed at the highest combination concentration (45 μg/mL MC-LR + 0.3 μg/mL CYN), which could lead to the consequences previously described. To our knowledge, this is the first report concerning these toxicological parameters, obtaining different responses after the exposure to the combination and to the isolated toxins, highlighting the importance of considering more realistic exposure-scenarios. Therefore, further investigations would be needed to clarify the effects of the MC-LR-CYN-combination on neuronal cells, and in different experimental models.

5. Conclusions

Our results showed a cytotoxic effect caused by the exposure to MC-LR and CYN individually and in combination in both undifferentiated and differentiated SH-SY5Y cells. CYN resulted more cytotoxic than MC-LR, but the combination presented the highest cytotoxicity. However, the isobolograms method establishes that these toxins together induce, mainly, an antagonistic response. Concerning oxidative stress biomarkers, only MC-LR and the combination decreased GHS levels at the highest concentration assayed. Moreover, AChE activity also showed different results for individual toxins and their combination. The morphology study corroborated those results observed with the cytotoxicity assays, since cell death by apoptosis was observed at almost all the concentrations assayed of both toxins and the combination. Taking all this into account, both cyanotoxins seem to present neurotoxic effects in the SH-SY5Y cell line. Thus, as the potential neurotoxicity induced by MC-LR and CYN is of interest, more studies concerning the different mechanisms by which both cyanotoxins can cross the blood-brain barrier (diffusion, transporters, direct affectation of the barrier, etc.) would be required. In addition, the affinity of these two cyanotoxins by the different cells composing the nervous system would be also of interest, together with the study of the toxic mechanisms these cyanotoxins can exert and their changes when single and combination toxin exposure are considered.

Uncited reference

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CHAPTER 2

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NEUROTOXICITY INDUCED BY MICROCYSTINS AND

CYLINDROSPERMOPSIN: A REVIEW

Review

Neurotoxicity induced by microcystins and cylindrospermopsin: A review

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A R T I C L E   I N F O

A B S T R A C T

Microcystins (MCs) and cylindrospermopsin (CYN) are among the most frequent toxins produced by cyanobacteria. These toxic secondary metabolites are classified as hepatotoxins and cytotoxin, respectively. Furthermore, both may present the ability to induce damage to the nervous system. In this sense, there are many studies manifesting the potential of MCs to cause neurotoxicity both *in vitro* and *in vivo*, due to their probable capacity to cross the blood-brain-barrier through organic anion transporting polypeptides. Moreover, the presence of MCs has been detected in brain of several experimental models. Among the neurological effects, histopathological brain changes, deregulation of biochemical parameters in brain (production of oxidative stress and inhibition of protein phosphatases) and behavioral alterations have been described. It is noteworthy that minority variants such as MC-LF and -LW have demonstrated to exert higher neurotoxic effects compared to the most studied congener, MC-LR. By contrast, the available studies concerning CYN-neurotoxic effects are very scarce, mostly showing inflammation and apoptosis in neural murine cell lines, oxidative stress, and alteration of the acetylcholinesterase activity *in vivo*. However, more studies are required in order to clarify the neurotoxic potential of both toxins, as well as their possible contribution to neurodegenerative diseases.

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1. Introduction

Cyanobacteria are a group of Gram-negative prokaryotes capable of growing under almost every environmental condition (Chorus and Bartram, 1999). Due to climate change and anthropogenic activities, their presence is increasing (Davis and Gobler, 2016). As a consequence, there is an enhancement of the production of toxic secondary metabolites of great importance for the ecotoxicology known as cyanotoxins (Duy et al., 2000). These toxins are classified as hepatotoxins (*e.g.* microcystins, nodularins), cytotoxins (*e.g.* cylindrospermopsin), neurotoxins (*e.g.* anatoxin-a, homoanatoxin, saxitoxins), dermatotoxins (*e.g.* lungbyatoxin) or irritant toxins (*e.g.* lipopolysaccharides) (Testai et al., 2016). There are different exposure routes for cyanotoxins, being the most important the oral route. In fact, many aquatic organisms are able to live in presence of cyanotoxins, and some of them have proved to bioaccumulate these secondary metabolites, acting as a reservoir for animals higher up the trophic chain, and also for humans (Berry and Lind, 2010; Gutiérrez-Praena et al., 2013). However, dermal, inhaling or even parenteral exposures are also possible (Buratti et al., 2017). Thus, the variety of targets and exposure routes together with the rise of cyanobacterial proliferations make of cyanotoxins a serious concern for animal livestock, human activities and public health (Testai et al., 2016).

In the last decades, the toxic effects of cyanotoxins on the nervous system have been widely studied, not only those caused by the so-called neurotoxins with well-defined mechanisms of action in this system such as anatoxins (ATX) and saxitoxins (STX), but also, by other cyanotoxins with different target organs (Florczyk et al., 2014). Neurotoxicity could be described as 'any adverse effect on the central or peripheral nervous system caused by chemical, biological or physical agents' (Costa et al., 2008). The keys to the brief communication within the nervous system are the generation of a potential of action as a quick response of dendrites to the neurotransmitters released from contiguous neurons, and its fast travelling for the neuronal axon for its release afterwards (Kem, 2000).

In this sense, among all the cyanotoxins, microcystins (MCs) and cylindrospermopsin (CYN) have proven to exert damage in the nervous system as well, in spite of not being considered as neurotoxins *per sé*. These are very common cyanotoxins (Table 1) able to put health at risk due to their ubiquity (Gutiérrez-Praena et al., 2013), as previously demonstrated in different human poisoning cases. The most serious episode associated with human exposure to MCs occurred when 126 people were intoxicated at a haemodialysis clinic in Caruaru (Brazil), causing the death of almost half of them. All patients presented malaise, weakness, dizziness, vertigo, tinnitus, mild deafness and, in severe cases, visual disturbance and blindness, grand mal convulsions, and gastrointestinal and hepatic symptoms (Pouria et al., 1998; Carmichael et al., 2001). Most of these symptoms have a neuronal origin, standing out the possible MCs-crossing the blood-brain barrier (BBB) as several authors have reported (Feurstein et al., 2009, 2010, 2011; Zhao et al., 2015a), causing their toxic effects.

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Table 1
Properties and environmental concentrations of some MCs congeners and CYN.

Toxin	Chemical structure	Molecular properties				Environmental concentration
		Molecular weight $[M+H]^+$	Molecular composition	Kow	BCF Plants	In surface waters (µg/L)
MC-LR	Cyclo(−D-Ala-L-**Leu**-D-erythro-β-methylAsp(isolinkage)-L-**Arg**-Adda-D-Glu(isolinkage)-*N*-methyldehydro-Ala	995.5561	$C_{49}H_{75}N_{10}O_{12}$	2.16 (Ward and Codd, 1999)	Up to 680.05±40.88 (Romero-Oliva et al., 2014)	Up to 2100 Faassen and Lürling (2013)
MC-LF	Cyclo(−D-Ala-L- **Leu** -D-erythro-β-methylAsp(isolinkage)-L-**Fe**-Adda-D-Glu(isolinkage)-*N*-methyldehydro-Ala	986.5234	$C_{52}H_{72}N_7O_{12}$	3.56 (Ward and Codd, 1999)	nf	Up to 51 (Graham et al., 2010)
MC-LW	Cyclo(−D-Ala-L- **Leu** -D-erythro-β-methylAsp(isolinkage)-L-**Trp**-Adda-D-Glu(isolinkage)-*N*-methyldehydro-Ala	1025.5343	$C_{54}H_{73}N_8O_{12}$	3.46 (Ward and Codd, 1999)	nf	Up to 260 (Faasen and Lurling 2013)
MC-RR	Cyclo(−D-Ala-L- **Arg** -D-erythro-β-methylAsp(isolinkage)-L- **Arg** -Adda-D-Glu(isolinkage)-*N*-methyldehydro-Ala	1038.5731	$C_{49}H_{76}N_{13}O_{12}$	1.54 (Liang et al., 2011)	Up to 54.09±17.01 (Romero-Oliva et al., 2014)	Up to 16,000 (Graham et al., 2010)
MC-YR	Cyclo(−D-Ala-L-**Tir**-D-erythro-β-methylAsp(isolinkage)-L- **Arg** -Adda-D-Glu(isolinkage)-*N*-methyldehydro-Ala	1045.5353	$C_{52}H_{73}N_{10}O_{13}$	nf	nf	Up to 343 (Simiyu et al., 2018)
CYN	2,4(1*H*,3*H*)-Pyrimidinedione, 6-[(*R*)-hydroxy[2a*R*,3*S*,4*R*,5a*R*,7*S*)-2,2a,3,4,5,5a,6,7-octahydro-3-methyl-4-(sulfooxy)-1*H*-1,8,8b-triazaacenaphthylen-7-yl]methyl]-, rel-(−)- (9CI)	416.1234	$C_{15}H_{21}N_5O_7S$	Highly water-soluble	Up to 3.88±0.33 (Cordero-Araújo et al., 2017)	Up to 800 (Shaw et al., 2000)

Abbreviations: BCF: bioconcentration factor; d.w: dry weight; Kow: octanol/water partition coefficients; nf: not found;

In the case of CYN, the most important outbreak occurred in Palm Island (Australia) in 1979, when 146 people were hospitalized with symptoms of malaise, vomits, anorexia, and hepatomegaly after drinking from a water supply that contained a CYN-producing *Cylindrospermopsin raciborskii* strain (Bourke et al., 1983; Griffiths and Saker, 2003). However, it is important to mention that CYN was also present in the Caruaru outbreak, possibly contributing to the neurological affectation reported (Bláha et al., 2009) although it is hard to differentiate the effects caused for each toxin in the symptoms observed, as both toxins are often present together in nature (Gkelis and Zaoutsos, 2014; Trainer and Hardy, 2015; Loftin et al., 2016; Buratti et al., 2017). Due to the low molecular weight of CYN, it might be able to cross the BBB. In fact, CYN was detected in brains of two fish species (Guzmán-Guillén et al., 2015; da Silva et al., 2018). Thus, although not being considered as neurotoxins, both cyanotoxins have demonstrated its neurotoxic potential in different *in vitro* and *in vivo* experimental models, increasing the interest of the scientific community in this matter. Taking into account all these facts, the aim of the present work was to gather the existent knowledge about the potential to exert neurotoxic effects of both toxins from 1998 to 2018.

2. Microcystins

Microcystins (MCs) are cyclic heptapeptides molecules containing a hydrophobic C_{20} D-amino acid commonly known as ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), crucial for the toxicity of these cyanotoxins due to their interaction with protein phosphatases (Song et al., 2006) (Fig. 1). >246 isoforms

of MCs have been detected (Spoof and Catherine, 2017), mainly differing in the L-amino acids at positions 2 and 4, causing differences in toxicokinetic and toxicodynamic properties (Rinehart et al., 1994). These compounds are the most widespread cyanobacterial toxins detected in freshwaters (Spoof and Catherine, 2017), being many the cyanobacteria genera capable of synthesize them: *Microcystis, Plankthotrix, Anabaena, Nostoc, Aphanizomenon, Anabaenopsis*, *Rivularia and Fisherella,* among others (Sivonen and Jones, 1999; Rao et al., 2002; Carey et al., 2007; Bittencourt-Oliveira et al., 2014; Cirés et al., 2014).

The most known mechanism of action of MCs is the protein serine/threonine phosphatases inhibition, able to cause phosphoprotein-deregulation, which leads to tumor promotion and apoptosis (MacKintosh et al., 1990; Vichi et al., 2016). Furthermore, the potential of MCs to increase reactive oxygen species (ROS) and to reduce glutathione (GSH) levels, causing oxidative stress and, therefore, apoptosis, has already been demonstrated (Puerto et al., 2011; Wang et al., 2013; Li et al., 2015a, 2015b; Liu et al., 2016; Qian et al., 2018). Although being considered as hepatotoxins, MCs can damage other organs such as intestines, heart or kidneys (Moreno et al., 2003; Atencio et al., 2008; Qiu et al., 2009; Li et al., 2011a; Zeng et al., 2014). In this sense, it has been demonstrated that MCs require organic anion transporting polypeptides (OATPs for humans/Oatps for rodents) in order to cross cell membranes (Chen and Xie, 2016). The OATP1B1 and OATP1B3 are common in liver cells, while OATP1A2 is thought to be the responsible for the transport of MC-LR, across the BBB and the kidneys, for example (Fischer et al., 2005; Feurstein et al., 2009). This means that significant amounts of MCs could reach
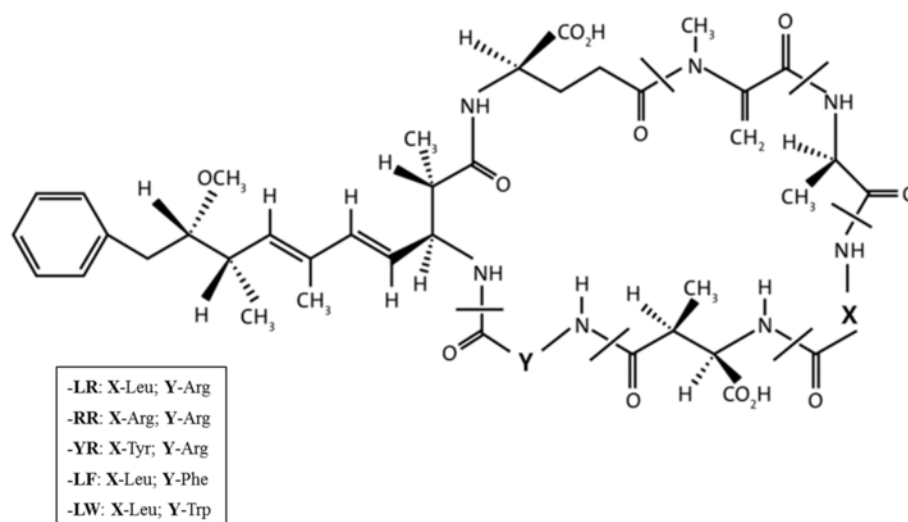
Fig. 1. Structure of MCs.

the brain across the BBB and induce brain pathology, depending on the type and expression of OATPs/Oatps at the BBB, the blood-cerebrospinal fluid barrier, and the neuronal cell membrane (Bronger et al., 2005; Huber et al., 2007; Westholm et al., 2009). Most of the existent studies have been carried out using MC-LR, due to its major presence and its wide demonstration of causing neurotoxic effects in several experimental models, although other more toxic congeners such as MC-LW and MC-LF have also been studied (Feurstein et al., 2009, 2010, 2011; Rozman et al., 2017). This can be due to the hydrophobicity of MC-LF and MC-LW. Structure variations and differences in molecular properties such as hydrophilicity/hydrophobicity can lead to a modification on molecular interactions with lipid membranes (Vesterkvist and Meriluoto, 2003) modifying PP-inhibitory activity (Díez-Quijada et al., 2018).

Concerning to their effects in the nervous system, Florczyk et al. (2014), Hu et al. (2016) and Mello et al. (2018) have reviewed the main mechanisms of neurotoxicity of MCs at different levels. Firstly, neurotransmission, by causing effects on GABAergic neurons. Secondly, neurochannels, by affecting the ionic concentrations in and outside the cells. Linked to this, signal transduction, as a consequence of the deregulation of Ca^{2+}, which, by activating calcineurin leads to apoptosis. Moreover, the production of oxidative stress, by deregulating several antioxidant enzymes such as catalase or superoxide dismutase (SOD). And finally, cytoskeleton disruption, by alteration of

structural brain proteins such as Tau. However, important contributions have been made lately, confirming these mechanisms using mostly *in vivo* experimental models. In this sense, the studies carried out using different animal models (mice, fish) revealed an important effect on the neurotransmission induced by MC-LR (Wu et al., 2016; Qian et al., 2018; Shin et al., 2018; Wang et al., 2018), together with an enhancement in oxidative stress in mice (Shin et al., 2018; Wang et al., 2018), and cytoskeleton disruption in the case of rats (Zhang et al., 2018) (Fig. 2).

2.1. Neurotoxicological in vitro studies performed with microcystins

Table 2 shows the different *in vitro* assays performed with MC-LR and some other congeners in different neuronal cell lines and primary cultures. The *in vitro* studies are relatively recent, comprising a range of ten years (2009–2018) (Table 2). This fact demonstrates the importance that MCs have lately acquired concerning their neurotoxicity nowadays. Thus, it is possible to find different studies carried out in permanent cell lines (PC12, BV-2, N2a, GT1–7, and SH-SY5Y) and in several primary cell cultures. It is also important to remark that all the toxins used in these studies are commercial standards with a purity >95%, which guarantees that the results reported are due to the MC itself and not to other potential bioactive compounds that can be present in cyanobacterial extracts (Falconer, 2007). Furthermore, it is

Fig. 2. Main mechanisms of neurotoxic action of MCs.

Table 2

In vitro neurotoxicity studies after exposure to MCs.

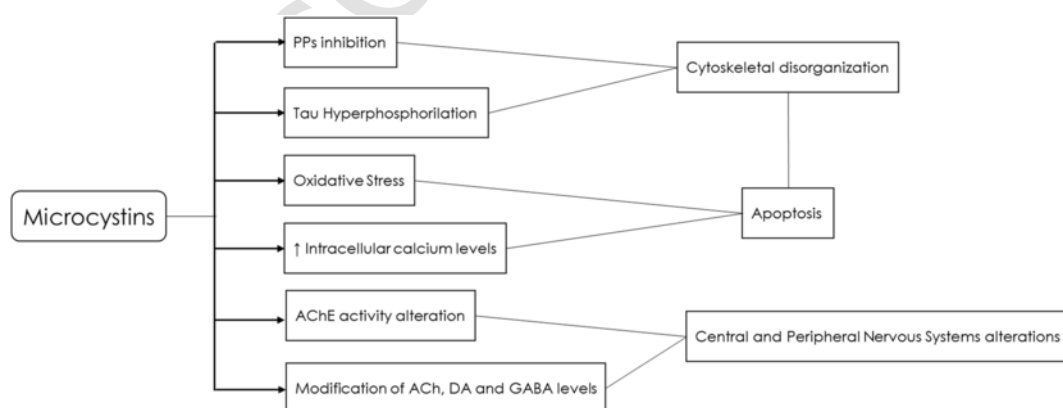| Toxin	Experimental model	Experimental conditions	Assays Performed	Relevant results	LC$_{50}$	References
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine WBC	0, 0.2, 0.4, 0.6, 0.8, 1, 3 and 5 μM for 48 h	MTT assay PPI assay	At 5 μM, complete loss of cell viability by MC-LF, decrease of cell viability by MC-LR and MC-LW (54% and 33%, respectively). Decrease of cell viability after exposure to -LF, -LW and -LR to ≥200 nM, ≥ 400 nM and ≥ 600 nM, respectively.	>10 μM 3 μM aprox 3 μM aprox	Feurstein et al. (2009)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine neurons	0, 0.31, 0.63, 1.25, 2.5, and 5 μM for 48 h	PPI assay	20% inhibition of PP activity at low MCs concentrations. Decrease of activity at 2.5 μM by 25% (−LR), 30% (-LW), and 60% (-LF). Decrease of PP activity by 65% at 5 μM -LF.	–	Feurstein et al. (2010)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary murine CGNs	0, 0.2, 0.4, 0.5, 0.6, 0.8, 1, 3, 5 and 10 μM for 48 h	MTT assay Apoptosis Morphology PPI assay Tau phosphorylation	At 5 μM, decrease of cell viability by -LR (to 70%), -LW (to 50%) and -LF (to 8%). 3–5 μM -LF caused the highest level of apoptosis. Apoptotic nuclei at 5 μM -LW while -LR did not induced them at any concentration assayed. Enhance of caspase-3/7 activity for -LF and -LW, and no changes for -LR. -LF caused a complete disintegration of the neurite network, whereas -LR induced a slight impairment. No statistical differences in PPI of -LR, while -LF induced a concentration-dependent inhibition from 2.5 μM. Tau phosphorylation fast and potent for -LF, being less evident for -LW, and with a low constant signal for -LR.	>10 μM 5 μM 1.5 μM aprox	Feurstein et al. (2011)
Pure MC-LR	Differentiated PC12 cells	0, 0.1, 0.5, 1, 5 and 10 μM for 6 h	PPI assay Tau phosphorylation p38-MAPK activation Morphology	MC-LR caused a concentration-dependent significant inhibition of PP2A by 27.4% at low concentrations, by 36.5% at 5 μM and by 60.5% at 10 μM, leading to Tau hyperphosphorylation. Drastic enhance of p38-MAPK phosphorylation with 10 μM MC-LR. Loss of the regular filamentous distribution and decrease of tubulin and actin fibers in the cytosol, enhancing in the periphery.	–	Meng et al. (2011)
Pure MC-LR	Differentiated PC12 cells	0, 1, 2.5, 5, 7.5 and 10 μM for 24 h	ROS Tau phosphorylation p38-MAPK activation	MC-LR induced a concentration- and time-dependent alteration of intracellular ROS until 6 h of exposure, recovering to the baseline at 18 h. A Tau phosphorylation was observed from 1 h of exposure, reaching the highest effect at 3 h, and gradually decreasing to basal levels. Enhance of p38-MAPK activation from 1 to 24 h of exposure.	–	Meng et al. (2013)
Pure MC-LR	Primary hippocampal neurons	0, 0.1, 0.3, 1, 3, 10 and 30 μM for 24 h	MTT assay Calcium mobilization	Decrease of cell viability by MC-LR in a concentration-dependent way. Enhance of apoptotic and necrotic neurons number with 1 μM MC-LR. The toxin induced a concentration-dependent intracellular calcium mobilization.	10 μM aprox	Cai et al. (2015)
Pure MC-LR	Primary hippocampal neurons	0, 0.3 and 3 μM for 48 h	Proteome analysis CaN activity MTT assay LDH release	Alteration of 45 proteins implied in calcium-ion signal transduction, apoptosis, oxidative stress response, and cytoskeleton structure. Enhance of CaN levels. Decrease of cell viability at the highest MC-LR concentration assayed. Enhance of LDH release with the increment of the concentration.	–	Li et al. (2015a)
Pure MC-LR	BV-2 cells N2a cells	0, 0.1 and 10 μM for 24, 48 and 72 h	MTT assay	BV-2 cells exposed to MC-LR never reached LD$_{50}$ levels at any of the exposure times, but significant decrease of viability after 24 h at both MC-LR concentrations, and only at 10 μM after 48 and 72 h. Decrease of cell viability after exposure to both concentrations assayed after 24, 48 and 72 h in N2a cells.	>10 μM 10 μM aprox	Takser et al. (2016)
Pure MC-LR	GT1–7 cells	0, 0.01, 0.05, 0.1, 0.5 and 1 μM for 48 h	Toxin uptake CCK-8 test	Uptake of MC-LR into cells was confirmed by western-blot, since it covalently bound to the PP1 and PP2A catalytic subunits. Decrease of cell viability in a concentration-dependent way. No affectation when deprived from the Oatp1a5 transporter.	–	Ding et al. (2017)
Pure MC-LR Pure MC-LW Pure MC-LF	Primary rat astrocytes	0, 0.5, 2 and 10 μM for 24 h	MTT assay Apoptosis Immunocytochemistry Morphology	Intracellular localization of MCs using immunocytochemistry. Cytoskeletal disruption, decrease of cell viability and enhance of number of apoptotic cells after MC-LW and MC–LF exposure. MC-LR did not cause any of the alterations above.	–	Rozman et al. (2017)

Table 2 (*Continued*)

Toxin	Experimental model	Experimental conditions	Assays Performed	Relevant results	LC$_{50}$	References
Pure MC-LR	SH-SY5Y cells	0, 5 and 10 μM for 24 h	Toxin uptake Tau phosphorylation PPI assay LDH release	Uptake of MC-LR into cells confirmed by western-blot, using PP1 and PP2A catalytic subunits-antibodies. Enhance of Tau phosphorylation the concentration of accumulated MC-LR. The PP2A activity was inhibited in a concentration-dependent way. The highest MC-LR concentration caused cell dead, related to Tau phosphorylation.	–	Zhang et al. (2018)

Abbreviations: BV-2: cellosaurus cell line; CaN: calcineurin; CCK-8: cell counting kit-8 test; CGNs: cerebellar granule neurons; GT1–7: hypothalamic neuronal mouse cells 1–7; LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N2a: fast-growing mouse neuroblastoma cells; Oatp: organic-anion-transporting-polypeptide; p38-MAPK: P38 mitogen-activated protein kinases; PC12: pheochromocytoma of rat adrenal medulla; PP: protein phosphatase; PPI assay: protein phosphatase inhibition assay; ROS: reactive oxygen species; SH-SY5Y: *Homo sapiens* bone marrow neuroblast; WBC: whole brain cells.

important to highlight that no studies have been performed using extracts *in vitro*.

2.1.1. Cell viability studies after exposure to MCs

Cell death caused by MCs in neuronal cells has been studied by different assays. Occupying an important place in these studies are the cytotoxicity assays. As it can be observed in Table 2, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay, the lactate dehydrogenase (LDH) release assay, and the cell counting kit-8 (CCK-8) test have been used to explore the cytotoxicity of MC congeners in several neuronal cell lines. In primary cell lines, Feurstein et al. (2009) found that MC-LF, -LW, and -LR induced a concentration-dependent decrease of primary murine WBC when exposed to 0–5 μM MCs for 48 h, being MC-LF the most potent toxin. Rozman et al. (2017) also evidenced the different cytotoxicity induced by the MC-LW and MC-LF congeners in primary rat astrocytes exposed to 0–10 μM MCs for 24 h. However, these authors did not find any significant reduction of viability in cells exposed to MC-LR. On the contrary, Cai et al. (2015) found a concentration-dependent reduction of cell viability in primary hippocampal neurons, although the MC-LR concentrations used were higher (0–30 μM) than in the previous study. Despite this, Li et al. (2015a) used lower concentrations of MC-LR (0–3 μM) in the same cellular model, remarking that they only found a reduction of cell viability at the highest concentration assayed after 48 h of exposure. These same authors also evaluated the LDH release, showing that this release increased with the MC-LR concentration. However, Zhang et al. (2018) observed that only the highest concentration (10 μM MC-LR) induced a significant loss of viability in SH-SY5Y cells exposed for 24 h. This fact would indicate that the cellular model could play a role in the MC-LR toxicity, being more sensitive those cells derived from the hippocampus.

Concerning permanent cell lines, different patterns have been observed. Thus, Takser et al. (2016) found that murine microglial BV-2 cell line suffered a decrease in cell viability when exposed to 0–10 μM MC-LR during 72 h. Furthermore, these same authors revealed that the N2a cell line presented an even more significant reduction of viability after 72 h of exposure, establishing possible differences between cells from different origins. The results obtained by Ding et al. (2017) were especially remarkable, finding that MC-LR induced a concentration-dependent reduction of viability in GT1–7 cells exposed up to 1 μM MC-LR during 48 h. In this study, the MC-LR concentrations used were pretty lower than those used by the rest of the authors. Thus, the main target of MC-LR in the nervous system seems to be the limbic system, since cells from hypothalamus and hippocampus have proven to be the most sensitive.

2.1.2. Effects of MCs in different proteins

Many of the presented studies deal with the fact that MCs need to enter the neuronal cells to exert their toxic effects. It is well known that MCs use OATP/Oatp to get into cells. In this sense, Feurstein et al. (2009) stated that primary murine whole brain cells (WBC) presented, at least, five Oatps, and demonstrated the role of these transporters in the toxicity induced by different MC congeners. Lately, the same authors employed primary murine neurons and cerebellar granule neurons (Feurstein et al., 2010, 2011), and demonstrated that MC-LF, -LW, and –LR produced a significant PPs inhibition at different concentrations, being MC-LF the most potent toxin and MC-LR the least one. Concerning MC-LR, Meng et al. (2011), in differentiated rat neuroendocrine PC12 cells, and Zhang et al. (2018), in human neuroblastoma SH-SY5Y cells, found that this toxin inhibited the PP2A in a concentration-dependent manner. However, MCs uptake has been also shown by imaging techniques. Thus, Rozman et al. (2017) confirmed the uptake of different MCs congeners by immunochemistry in primary rat astrocytes. Moreover, Ding et al. (2017) and Zhang et al. (2018) used the western-blot technique to demonstrate the penetration of MC-LR in hypothalamic neuronal mouse cells 1–7 (GT1–7) and SH-SY5Y cells, respectively, analyzing the PP1 and PP2A catalytic subunits, which appeared reduced as the toxin concentration increased.

Inhibition of PP2A activity has been described as the main toxic mechanism of MCs (Yoshizawa et al., 1990), which is related to the selective destruction of microtubules, leading to cell death. Different proteins are involved in cellular organization, and among them, one of the most relevant is Tau. This abundant microtubule-associated protein which main function to stabilize the microtubules assembly, is less effective the more phosphorylated Tau is (Buée and Delacourte, 2001), being associated with microtubule dysfunction and cell death (Feurstein et al., 2011). These last authors found, in primary murine cerebellar granule neurons (CGNs), that MC congeners induced Tau hyperphosphorylation at lower concentrations than the needed for PP2A inhibition, which could evidence that specific proteins from the nervous system display more sensitive response to MCs. However, these concentrations did not lead to significant cell death by apoptosis (activation of caspase-3/7 was absent); although disruption of the neurite network was observed, which is in agreement with the findings of Rozman et al. (2017) in primary rat astrocytes. Meng et al. (2011) also established the connection between the inhibition of PP2A and Tau protein hyperphosphorylation in differentiated PC12 cells. Furthermore, these authors studied afterwards Tau phosphorylation through the p38-mitogen-activated protein kinase (p38-MAPK), reporting that MC-LR exposure induced p38-MAPK activation, although at higher concentrations than those required for the inhibition of PP2A. Thus, they established that this could be an indirect mecha-

nism of Tau hyperphosphorylation. In addition, they also found that the heat-shock protein 27 (HSP27), responsible of actin cytoskeleton remodeling, was also increased due to the activation of p38-MAPK, contributing to the cell disruption caused by MC-LR. Related to this, Meng et al. (2013) demonstrated that the previously described activation of p38-MAPK by MC-LR in PC12 cells was downstream of ROS-dependent signaling cascades. More recently, Zhang et al. (2018) confirmed the activation of the p38-MAPK in SH-SY5Y cells exposed to MC-LR. Moreover, these authors also found that MC-LR activates the c-Jun N-terminal kinase (JNK), a protein associated with the induction of cell death by apoptosis. Besides, MC-LR induced the phosphorylation of the glycogen synthase kinase-3 (GSK-3β), contributing to the dissociation of the regulatory subunit B55α from the PP2A and its degradation, facilitating Tau hyperphosphorylation.

2.1.3. Involvement of MCs in the $[Ca^{2+}]i$ levels

Intracellular calcium ($[Ca^{2+}]_i$) levels are crucial for cell survival. In this sense, Ding et al. (2001) indicated that MC-LR is implicated in Ca^{2+} release from mitochondria and the activation of Ca^{2+}/calmodulin-dependent protein kinase, which triggers cell death by apoptosis in hepatocytes. Thus, Cai et al. (2015) found a concentration-dependent Ca^{2+} mobilization in primary hippocampal neurons exposed to 0–30 μM MC-LR. These authors demonstrated that the increase of $[Ca^{2+}]_i$ levels could be due mainly to its mobilization from the endoplasmic reticulum. Mitochondria seemed not to play an important role in the cascade of $[Ca^{2+}]_i$. This fact is in agreement with the results obtained in the previously described MTT assays (Feurstein et al., 2009, 2011; Takser et al., 2016), since authors described a concentration-dependent loss of cell viability, but only a few observed significant differences against the control groups. In addition, Li et al. (2015a) reported that MC-LR participated in the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (CaN), through the mobilization of $[Ca^{2+}]_i$ levels, leading to the activation of an apoptotic caspase cascade. In this sense, Feurstein et al. (2011) found that MC-LF and MC-LW induced a concentration-dependent increase of caspase-3/7 activity in primary murine CGNs. Furthermore, Rozman et al. (2017) also observed apoptosis in primary rat astrocytes exposed to different MC congeners. However, these authors did not propose any theory about the apoptosis pathway. These findings could be in agreement with the reports of Cai et al. (2015) and Li et al. (2015a) concerning Ca^{2+} mobilization and apoptosis.

Summarizing, MC-LW and –LF have proven to exert higher neurotoxic effects *in vitro* than MC-LR. However, since MC-LR is the most abundant congener in nature, all the studies presented in Table 2 have been carried out using this cyanotoxin. The way these toxins reach the nervous system is not fully elucidated yet, although several authors demonstrated the participation of OATPs/Oatps in their transport, together with an inhibition of the protein phosphatase. Once inside neuronal cells, MCs have shown to disrupt several proteins participating in the cellular structure (PP2A, Tau, p38 MAPK, HSP27, GSK-3β, *etc.*), inducing cytoskeleton remodeling and cell death. In addition, cellular disruption has been demonstrated as well by cytotoxicity and apoptosis assays. Both mechanisms could be associated with the increment of $[Ca^{2+}]_i$ levels. However, it is noteworthy that cells affected by MCs are mainly those present in the limbic system, pointing out this system as a possible target for MCs.

2.2. Neurotoxicological in vivo studies performed with microcystins in aquatic animals

Several works have investigated so far MCs potential neurotoxicity in different fish species, mainly in zebrafish (*Danio rerio*) (Table

3). The first studies reporting the chronic effects of dissolved MC-LR on the fish behavior were performed by Baganz et al. (1998, 2004). Behavioral studies are important to establish the lowest level of disturbance. In this sense, these authors observed that MC-LR induced a decrease of daytime and nighttime activity in *D. rerio* after their exposure to high concentrations, while at low ones, that reduction at night was compensated by a rise in their daytime activity. On the contrary, at high concentrations, *Leucaspius delineatus* reduced its activity during the daytime, increasing at night, whereas a rise was reported during both day and night at low concentrations. These compensative responses could be explained as an escape strategy or as a consequence of some changes in the spatial orientation to deal with alterations in the medium conditions, represented by the presence of MCs. However, the decreased motility observed at high MC-LR concentrations may be interpreted as an attempt to save energy, needed maybe to biotransform the toxin, which is a possible reason why glutathione-S-transferase (GST) activity appeared enhanced. *L. delineatus* showed greater sensitivity than *D. rerio*, as it responded earlier and for a longer period of time (Baganz et al., 2004).

Neurotoxicity of pure MC-LR at the proteomic level was firstly demonstrated in zebrafish brains after chronic exposure (30 days) by Wang et al. (2010) and in developing zebrafish larvae after 96 h post-fertilization exposure by Li et al. (2011b). Furthermore, chronic exposure seemed to interfere concomitantly with signal transduction, leading to apoptosis, transport and protein degradation, and increasing the PP activity at higher toxin concentrations by PP2Cα2 overexpression (Wang et al., 2010). Li et al. (2011b) suggested a potential involvement of creatine kinase (CK) and dihydropyrimidinase-like 2 (DRP2) in the neurotoxicity induced by MC-LR, which were upregulated in larvae of zebrafish. The CK seemed to be correlated with increased energy requirements, and DRP2 with axonal outgrowth, cell migration, neuronal growth, and pathfinding. In this sense, a decreased expression of DRP2 has been reported in schizophrenia, Alzheimer's disease, the Down syndrome, and affective disorders (Johnston-Wilson et al., 2000; Lubec et al., 1999).

Pavagadhi et al. (2012) studied the influence of sub-lethal concentrations of dissolved MC-LR and MC-RR (0–10 μg/L) on several oxidative stress parameters in the brain of zebrafish adults such as GST, glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) activities. Generally, most of the parameters followed a bell-shaped curve for both toxins, with peaks at different concentrations. Most of these enzyme activities rose at lower concentrations and decreased at the highest (5 and 10 μg/L). However, discrepancies between GPx and GR activities were observed as the effects of MC-LR were more prominent in GPx activity while GR activity was more enhanced after exposure to MC-RR. These variations could probably be due to the biochemical adaptive response of the organisms to MCs exposure depending on their specific toxicity.

A more recent study has shown that accumulation of MC-LR in zebrafish larvae led to hypoactivity with alteration of the cholinergic system, showed by decreased dopamine (DA) and ACh levels, and increased AChE activity, which could also yield to hypoactive muscular contraction and behavioral responses (Wu et al., 2016). In addition, and similar to previous works, their proteomic analysis suggested that this neurotoxicity could be related to neuron maturation, axon growth, and cytoskeleton regulation. Nevertheless, if these effects induced by MC-LR could be of parental transmission or not was later clarified by chronic exposures of adult zebrafish to environmentally relevant concentrations of MC-LR (1–25 μg/L), demonstrating, for the first time, the toxin accumulation and developmental neurotoxicity in offspring (Wu et al., 2017). The mechanisms by which these transgenerational effects are exerted could be by interrupting

Table 3

In vivo neurotoxicity studies in several aquatic animal models exposed to MCs.

Microcystin congener/ cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Aquatic animals					
Pure MC-LR	Zebrafish *(Danio rerio)*	0.5, 5 or 15 µg/L for 25 days and 50 µg/L for 6 days, by oral and transdermal route	Behavioral study	The motility showed a dose-effect relationship and changes in the circadian rhythm	Baganz et al. (1998)
Pure MC-LR	Zebrafish *(Danio rerio)* Sunbleak *(Leucaspius delineatus)*	0.5, 5 or 15 µg/L for 17 days and 50 µg/L for 6 days, by oral and transdermal route	Behavioral study	Lower concentrations increased motility, whereas the highest one decreased the activity of both species. *D. rerio* was less sensitive. Despite *D. rerio* remained diurnally active, the swimming activity of *L. delineatus* was altered, reversing diurnal and nocturnal activity	Baganz et al. (2004)
Pure MC-RR	Peppered catfish *(Corydoras paleatus)*	0.5, 2, 5 or 10 µg/L for 24 h, by oral and transdermal route	Oxidative stress parameters (GR, POD, GPx, CAT and LPO) and detoxification system (GST activity)	Increased LPO levels in brain of exposed fish, and a general activation of the antioxidant enzymatic system	Cazenave et al. (2006)
Pure MC-RR	Onesided livebearer *(Jenynsia multidentata)*	0.01, 0.1 or 1 µg/g for 24 h, by oral route	Swimming activity and detoxification system (GST activity)	Low doses increased swimming activity, while the highest dose headed to small reduction after 20 h	Cazenave et al. (2008)
Pure MC-LR	Zebrafish *(Danio rerio)*	2 or 20 µg/L for 30 days, by oral and transdermal route	Protein expression	Oxidative stress, dysfunction of cytoskeleton assembly and macromolecule metabolism, and interference with signal transduction and other functions in brain. The PP activity rose with MC-LR concentration	Wang et al. (2010)
Pure MC-LR	Zebrafish *(Danio rerio)*	0.2, 0.5, 2 and 5 mg/L at 96 hpf, by oral and transdermal route	Protein and gene expression	Upregulation of CKs and DRP2	Li et al. (2011b)
M. aeruginosa containing MC-LR	Rainbow trout *(Oncorhynchus mykiss)*	0.75, 1.8 and 5 µg/L for 96 h, by oral and transdermal route	Oxidative stress parameters (GST activity, LPO levels), PBP and AChE activity	Neither GSH activity nor LPO altered. Lower levels of PBP and AChE	Gélinas et al. (2012)
M. aeruginosa containing MC-LR	Zebrafish *(Danio rerio)*	50 or 100 µg/L for 24 h, by branchial and oral route	AChE activity and protein and gene expression of whole brain	Enhancement of the AChE activity depending on the exposure route	Kist et al. (2012)
Pure MC-LR and MC-RR	Zebrafish *(Danio rerio)*	0.1, 0.5, 1, 5 or 10 µg/L for 4, 7 and 15 days, by oral and transdermal route	Antioxidant enzymatic activities (GST, GPx, GR and SOD)	A bell shaped curve of response for most of the parameters	Pavagadhi et al. (2012)
Crude algae containing MC-RR	Goldfish *(Carassius auratus)*	0, 50 or 200 µg/kg b.w., tested at 6, 12, 24 and 48 h, by intraperitoneal injection	Glucose levels and antioxidant enzymatic activities (TAOC, SOD, CAT and GPx), histopathological study and protein and gene expression of globin proteins	The injection before hypoxia and reoxygenation reduced antioxidant capacity in most organs. Myoglobin and neuroglobin mRNAs were induced in the brain	Okogwu et al. (2014)
Planktothrix agardhii containing MC-YR and MC-LR *M. aeruginosa* containing MC-LR	Zebrafish *(Danio rerio)*	0.3, 1, 3 or 10 g d.w./L for 96 h, by transdermal and oral route	Behavioral study	Slight increase of movement in zebrafish embryos	Jonas et al. (2015)
Pure MC-LR	Zebrafish *(Danio rerio)*	0.8, 1.6 or 3.2 µg/L for 120 hpf, by transdermal and oral route	Developmental toxicity and locomotor study, ACh and DA levels, protein and gene expression related to development, AChE activity	Hypoactivity of larvae and alteration of the cholinergic system	Wu et al. (2016)

Table 3 (*Continued*)

Microcystin congener/ cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Pure MC-LR	Zebrafish *(Danio rerio)*	0.3, 3 or 30 μg/ L for 90 days, by transdermal and oral route	Histopathological study and protein and gene expression of GABA and glutamate	Edematous and collapsed myelinated nerve fibers, distention of endoplasmic reticulum and swelling mitochondria in brain	Yan et al. (2017)
Pure MC-LR	Zebrafish *(Danio rerio)*	1, 5 or 25 μg/L for 60 days, by transdermal and oral route	Behavioral study, protein and gene expression, levels of MC-LR, DA, GABA, serotonin, ACh and DOPAC, and AChE activity	Parental exposure resulted in MC-LR accumulation and developmental neurotoxicity in offsprings	Wu et al. (2017)
M. aeruginosa containing MC-LR	Zebrafish *(Danio rerio)*	0.02, 0.04 or 0.08 OD values, for 4 days, by transdermal and oral route	Locomotor behavioral study, gene expression, AChE and DA levels	Affectation of both cholinergic and dopaminergic systems changes in the gene transcription of the nervous system, and a decrease of the locomotor activity in larval zebrafish	Qian et al. (2018)

Abbreviations: ACh: acetylcholine; AChE: acetylcholinesterase; b.w.: body weight; CAT: catalase; CKs: creatine kinases; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; DRP2: dihydropyrimidinase-like 2; d.w.: dry weight; GABA: gamma-aminobutyric acid; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; hpf: hours post-fertilization; LPO: lipid peroxidation; OD: optical density; PBP: protein-bound phosphate; POD: guaiacol peroxidase activity; SOD: superoxide dismutase; TAOC: total antioxidant capacity.

the neuronal development and/or by hampering the neurotransmitter systems (as shown by decreases in DA and serotonin levels, and in AChE activity). Moreover, exposure of zebrafish embryos to similar concentrations of MC-LR for 90 days led to several histopathological damages in the brain (Yan et al., 2017). Despite lacking the clear cerebral cortex of higher vertebrates, fish cerebra rule complex behavior such as escaping from predators, swimming, and feeding modulation. Thus, it would make sense that the ultrastructural changes detected in this study could have impaired the function of nerve fibers in zebrafish exposed to MC-LR. These authors suggested that the disruption of the GABA pathway might be also implicated in the mechanism of MC-LR-induced neurotoxicity (Yan et al., 2017). The stress response in fish is regulated by the hypothalamic-pituitary-adrenal (HPI) axis, which modulates cortisol levels (Yan et al., 2012; Chen et al., 2016), having both important functions in behavior and development. In addition, cross-talk among the nervous, endocrine, and immune systems have been previously reported in fish (Steenbergen et al., 2011). In this sense, Okuzawa et al. (2003), Liu et al. (2015), Zhao et al. (2015b), Su et al. (2016) and Chen et al. (2018) observed altered transcription of genes along the HPI axis in zebrafish, mostly of gonadotropin hormone, which is also a modulator of the reproductive behavior. Moreover, Chen et al. (2018) observed, for the first time, that MC-LR altered cortisol levels. Thus, neurotoxicity of MCs could have an impact on endocrine disruption, influencing the autonomic nervous system activity.

Apart from *D. rerio* and *L. delineatus,* the effects of pure MC-LR have been also described in whitefish (*Coregonus lavaretus*). Thus, MC-LR induced an up-regulation of the protein expression of the glial fibrillary acidic protein (*gfap*), suggesting neuronal toxicity, although no changes were observed in the expression of MiR124-3p (Florczyk et al., 2018). Thus, after damage to the central nervous system (CNS), astrocytes normally act by reaction with a quick synthesis of *gfap*, whereas the most abundant microRNA in the nervous system, MiR124, is involved in brain development and neuronal regulation. These results provide new information to understand the role of microRNAs in the mechanisms of MC-LR-induced neurotoxicity, and they suggest that MiR124-3p cannot be considered as a biomarker of MC-LR-induced brain injury.

In agreement with the findings *in vitro*, Fischer et al. (2005) demonstrated, in oocytes of the frog *Xenopus laevis*, that human OAT-P1A2, expressed in endothelial cells of the BBB, mediates the transport of MC-LR into the brain. Furthermore, they do not rule out

that other transporters, as Oatp1c1/OATP1C1, may be also involved in this function.

Studies conducted with lyophilized cyanobacterial cultures containing MCs are scarcer compared to those performed with pure MCs. In this regard, Fischer and Dietrich (2000) detected, for the first time, MC protein-adducts in the brain of carp (*Cyprinus carpio*) acutely exposed to a freeze-dried culture of *M. aeruginosa* containing MC-LR, although no pathological changes were observed in brain. Later, Gélinas et al. (2012) studied several antioxidant parameters and AChE activity in brain after exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to crude extract from *M. aeruginosa* containing MC-LR (0–5 μg/L) for 96 h. No significant changes were observed in GST activity or in LPO levels, and a decrease in AChE activity only occurred at the highest concentration assayed. However, an evident reduction of the protein-bound phosphate at all concentrations assayed was found, which could lead to a diminishment of protein phosphatase activity. Contrarily, after acute exposure to MC-LR isolated from *M. aeruginosa* by dissolving the toxin in water and intraperitoneally, Kist et al. (2012) demonstrated that zebrafish brain suffered an increase of AChE activity only when dissolved, being relevant as its over-expression can promote apoptosis. According to the authors, AChE effect in brain may be indirectly caused by the calcineurin, present in the zebrafish brain. In agreement with Gélinas et al. (2012) but in discordance with Kist et al. (2012), Qian et al. (2018) reported a decrease in AChE levels in larvae of the same species after exposure to a *M. aeruginosa* culture containing MC-LR. This could have, as a consequence, a reduction of the gene transcription of *ache*, together with a concentration-dependent decline of the nicotinic acetylcholine receptor a-7 (*chrna7*) transcription, being this, at least, one of the possible causes of the slowing down of the swimming speed. Besides, neuronal development and differentiation effect, impaired synapse formation, astroglia effect and a concentration-dependent reduction of dopamine were observed; together with an effect of the dopaminergic system in the zebrafish larvae. Differences in locomotion were observed in the embryos of the same species exposed to *Planktothrix agardhii* containing MC-LR and MC-YR, and to *M. aeruginosa* containing MC-LR (Jonas et al., 2015).

The neurotoxic effects of pure MC-RR on aquatic organisms have been far less investigated in comparison to pure MC-LR, and they are somehow contradictory. Although Cazenave et al. (2006) reported the brain of *Corydoras paleatus* as the most affected organ after exposure

to dissolved MC-RR by increases on lipid peroxidation (LPO) levels and decreases in GST activity, they were not able to detect the toxin in brain of this species (Cazenave et al., 2005). In agreement with this study, Cazenave et al. (2008) found that exposure of *Jenynsia multidentata* to MC-RR led to oxidative stress and altered locomotor activity. The hyperactivity observed at low doses suggests an escaping from the stress of MC-RR exposure, while the reduced swimming activity together with the increased detoxification at higher doses may represent a reallocation of energy (Cazenave et al., 2008), response that was obtained as well in previous studies carried out, in this case, with MC-LR (Baganz et al., 1998, 2004). In addition, fish hyperactivity could be also a result of the alert reaction caused by the presence of MC-RR in the fish brain, showing for the first time that MC-RR, although being more hydrophilic than MC-LR, is able to cross the BBB in *J. multidentata* (Cazenave et al., 2005).

Up to date, only one study has evaluated the effect on the fish brain after exposure to MC-RR extracted from freeze-dried crude algae (Okogwu et al., 2014). *Carassius auratus* showed a reduction of the total antioxidant capacity in brain combined with a hypoxia-reoxygenation process. A decrease in the SOD and GPx levels was observed during reoxygenation, as myoglobin and neuroglobin were upregulated both during hypoxia and reoxygenation, which might help to the detoxification process of reactive nitrogen species and ROS, being of use in the fight against oxidative stress.

Generally, the effects of both pure MCs and those from cyanobacterial blooms have been shown in the central and peripheral nervous systems of several fish species, although different sensitivity was observed among them. Main observations were changes in behavior, oxidative stress parameters, genes involved in energy requirements and axonal growth, and in cholinergic and dopaminergic systems, together with disruption of the GABA pathway. These, together with MC-LR accumulation in fish brain and offspring, could explain the observed transgenerational changes and developmental neurotoxicity of MC-LR. Compensation responses in the circadian rhythm of fish have been also reported, with a generally increased activity at low doses and the opposite at high doses. In any case, the neurotoxic effects of MC-RR have been less investigated than those of MC-LR, in spite of being one of the most common congeners. More studies are needed to clarify the ability of MC-RR to cross the BBB in other aquatic species, given its differential detection in the two fish species studied. Moreover, comparative studies of the neurotoxicity induced by exposure to pure MCs or to cyanobacterial extracts could help to clarify MCs crossing of the BBB in aquatic organisms. The potential energy reallocation in the brain of MCs-exposed organisms also deserves further research, together with its effect on the endocrine system because of the damage caused in the HPI axis. Furthermore, investigating the inhibition of OATP-mediated MCs transport could be of interest to provide an option for neurotoxicity prevention.

2.3. Neurotoxicological in vivo studies performed with microcystins in terrestrial animals

Nowadays, several *in vivo* studies have been carried out focusing on the neurotoxic potential MCs can exert in terrestrial animals (Table 4). Many of them have been performed in nematodes (Li et al., 2009a, 2009b; Ju et al., 2013, 2014; Moore et al., 2014; Saul et al., 2014), mice (Shin et al., 2018; Wang et al., 2018) and rats (Li et al., 2012a, 2012b; Wang et al., 2013; Li et al., 2014; Li et al., 2015b; Zhang et al., 2018) using pure MC congeners, mainly MC-LR. This is probably due to the fact that, although a total of 246 variants of MCs have been described so far (Meriluoto et al., 2017), MC-LR has demonstrated to be one of the most toxic structural variants, con-

tributing on 46–99.8% of the total MCs in natural waters (Ufelmann et al., 2012). Considering that cyanotoxins are not found isolated in nature but together with other substances produced in cyanoblooms, very few studies have been conducted using cyanobacterial biomass cultures or their extracts for terrestrial animal exposure (Pašková et al., 2008; Wang et al., 2008; Ju et al., 2014; Zhao et al., 2015a, 2015b).

Approximately a third part of these studies have been performed using the nematode *Caernorhabditis elegans* as experimental model and almost under the same experimental conditions. This may be due to its short lifespan and its usage as an environmental bio-indicator, reacting to a variety of environmental stimuli (Mutwakil et al., 1997; Graves et al., 2005). Moreover, *C. elegans* only presents 302 neurons, and the complete writing diagram for chemical and electrical connections is available (White et al., 1986). It is also important to highlight that, as a liver-lacking animal, the neurotoxic effects were more obvious (Saul et al., 2014).

The first study performed in *C. elegans* exposed to pure MC-LR reported a decrease in the chemotaxis to NaCl and diacetyl and in the thermotaxis in a concentration-dependent manner, suggesting damage on the corresponding sensory neurons (Li et al., 2009a). These effects were probably caused by the disruption of ASE and AWA sensory neurons, responsible for the chemotaxis, while an impairment of sensory neurons AFD and interneuron AIY, responsible for the thermotaxis, was reported as well (Satterlee et al., 2001; Li et al., 2009a), demonstrating a genetic control of these neurons by MC-LR. According to these results, Li et al. (2009b) reported a significant decrease of lifespan and body size after exposure to the highest concentrations of MC-LR assayed, together with a decrease of the head thrash and body bend after exposure to low concentrations. Moreover, effects on generation time, brood size and stress parameters were also observed. Ju et al. (2013) reported that low concentrations of MC-LR produced a significant decrease of body bend and head thrash frequency after 8 h of exposure while, after 24 h, all concentrations did, showing a time-dependent response. Moreover, the morphology effects caused by different neurotransmitters after exposure to MC-LR were evaluated and, although no structural alterations were observed in the cholinergic, serotonergic, dopaminergic and glutamatergic systems, a GABAergic neuronal loss and aberrant neuronal morphology were observed after exposure to the highest concentration of MC-LR. Furthermore, this study revealed that MC-LR induced 1) adverse effects on the transportation and location of GABA altering unc-47, unc-46, and unc-30 gene expression and 2) alteration of both the inhibitory and excitatory GABA receptors decreasing unc-49 and exp-1 expression levels. This effect on GABA could lead to the effects previously observed in the locomotor behavior. In agreement with these results, Ju et al. (2014) reported a significant decrease of different autonomic functions, such as body bend and touch response, move length, pharyngeal pumping frequency and defecation period interval (only after 24 h of exposure to the highest concentration of MC-LR). These authors demonstrated, exposing to a filtrate of *M. aeruginosa* culture containing MCs, that the response opposed to the one obtained with pure MC-LR, observing an increase in locomotive behavior and pumping activity and no alteration of sensory functions. These differences could be due to 1) the higher concentration present in the biomass compared to pure MC-LR used (300 *vs* 100 μg/L), 2) the presence of other active substances, and 3) the presence of several MC congeners, such as MC-RR and MC-YR. In addition, Moore et al. (2014), demonstrated alteration to diacetyl after exposure to MC-LR, showing an alteration of the function of the AWA sensory neuron. However, the effects on the chemotaxis to benzaldehyde after exposure to MC-LR, regulated by AWC neurons, was not observed, high-

Table 4
In vivo neurotoxicity studies in different terrestrial models exposed to MCs.

Microcystin congener/ cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Nematodes					
Pure MC-LR	*Caenorhabditis elegans*	1, 10, 20, 40, 80 or 160 µg/L for 24 h in sterile culture plates	Behavioral study and gene expression	Decrease of chemotaxis to NaCl and diacetyl from 40 µg/L Decrease of thermotaxis from 20 µg/L Decrease of expression patterns of sensory neurons (ASE, AWA, AFD and AIY)	Li et al. (2009a)
Pure MC-LR	*Caenorhabditis elegans*	1, 10, 20, 40 or 80 µg/L in different periods of time, in sterile culture plates	Behavioral study, morphological changes, gene expression and life-cycle indices	Decrease of head thrash, body bends Decrease of body size Enhance of *gfp* gene expression Decrease of life span, brood size, generation time	Li et al. (2009b)
Pure MC-LR	*Caenorhabditis elegans*	0.1, 1, 10 or 100 µg/L for 8 or 24 h, in 12-well sterile culture plates	Behavioral studies, morphologic changes and gene expression	Decrease of locomotion behavior Enhance of neuronal loss of GABAergic neurons, presenting aberrant neuronal morphology at 10–100 µg/L No changes in cholinergic, serotonergic, dopaminergic and glutamatergic neurons Decrease of Gene expression affecting GABAergic neurons	Ju et al. (2013)
Pure MC-LR *Microcystis aeruginosa* culture containing MC-LR, MC-RR and MC-YR	*Caenorhabditis elegans*	0.1, 1, 10 or 100 µg/L for 24 or 72 h, in sterile culture plates 300 µg/L for 24 or 72 h, in sterile culture plates	Behavioral study	Decrease of body bends, move length, pharyngeal pumping frequency and touch response Alteration of the thermotactic behavior after 72 h of exposure to 100 µg/L Enhance of motile and pumping activity	Ju et al. (2014)
Pure MC-LR Pure MC-LF	*Caenorhabditis elegans*	1, 10, 40, 80, 160, 320, 500, or 1000 µg/L for 24 h, in sterile culture plates 1, 10, 100, 160 or 320 µg/L for 24 h, in sterile culture plates	Function of sensory neurons	Affectation of AWA sensory neurons, but not of AWC sensory neurons: MC-LF > MC-LR	Moore et al. (2014)
Pure MC-LR	*Caenorhabditis elegans*	1, 50 or 100 µg/L in different periods for life cycle, in sterile culture plates	Life-cycle indices and gene expression	Decrease of lifespan, body length and brood size after 100 µg/L of exposure Alteration of genes expression after 100 µg/L of exposure	Saul et al. (2014)
Birds					
Cyanobacterial biomass containing MC-LR, MC-RR, MC-YR and MCs similar compounds	Japanese quail (*Coturnix coturnix japonica*)	0.045, 0.459, 4.605 or 46.044 µg/day for 10 or 30 days, by oral route	Oxidative stress parameters	Alteration in brain, after acute exposure: Decrease of GSH, Enhance of TBARS, Enhance of EROD. After subchronic exposure: Enhance of GSH, Enhance of GPx Enhance of TBARS, Enhance of EROD	Pašková et al. (2008)
Mammals					
MC raw extracts containing mainly [D-Leu[1]]MC-LR	Rats	1 µL of extracts containing 0.01 or 20 µg/L (equivalent to 0.045x10E-6 and 9.1x10E-5 µg/kg) by intrahippocampal injection	Behavioral study, oxidative stress parameters and DNA damage	Enhance of latency of long-term memory in rats exposed to 20 µg/L Decrease of latency of memory retrieval Enhance of working and reference memory errors after 8 days of exposure Enhance of GST activity in brain rats exposed to 0.01 µg/L Enhance of LPO content in brain rats exposed to 20 µg/L DNA damage in brain of both MCs doses treated rats	Maidana et al. (2006)
Extracted and purified MC-LR and MC-RR from blooms	Rats	80 µg MC-LReq/kg b.w. injected i.v. The analysis was performed 1, 2, 4, 6, 12 and 24 h post-injection	Determination of MCs content in different tissues by LC-MS	MCs contents in brain (0.2%): 2 > 24 > 1 > 12 > 6 ≈ 4 h post-injection kidney > lung > stomach > liver > small intestine > gonad > spleen > muscle > heart > **brain**	Wang et al. (2008)
Pure MC-LR	Rats	1 µL containing 1 or 10 µg/L MC-LR (equivalent to 5x10E-6 or 5x10E-5 µg/kg), bilaterally injected into hippocampal. Parameters were measured 15 days post-injection	Behavioral study, histopathological study and oxidative stress parameters	Enhance of latencies to find the platform Decrease of swimming distance in the target zone Swimming speed did not change Decrease of total hipocccampal neurons Highest MC-LR dose: Enhance of LPO, Enhance of CAT, Enhance of GPx, Enhance of SOD Lowest MC-LR dose: Enhance of LPO, Enhance of CAT	Li et al. (2012a)

Table 4 (*Continued*)

Microcystin congener/ cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
Pure MC-LR	Rats	1 or 10 μg/kg day i.p. injected for 50 days	Behavioral study, histopathological study, protein expression, MC-LR content analysis	Enhance of latencies to find the platform. Decrease of swimming distance in the target zone. Enhance of degeneration and apoptosis of hippocampal cells. Hyperphosphorylation of tau. 41.6 ± 8.45 ng/g d.w. of MC-LR was detected in brain of rats exposed to 10 μg/kg day	Li et al. (2012b)
Pure MC-LR	Rats	10 μL containing 5 or 25 μg/L (equivalent to 2.5x10E-4 or 1.25x10E-3 μg/kg), by i.c.v. injection MC-LR+LiCl and SB216763 inhibitors of GSK-3β	Electrophysiologycal studies	Enhance of PPs activity. Decrease of phosphorilated GSK-3β. Decrease of LTP. Inhibitors avoid effects produced by MC-LR	Wang et al. (2013)
Pure MC-LR	Rats	0.2, 1 or 5 μg/kg every 2 days for 8 weeks, by intragastric route	Behavioral study, histopathological study and immunohistochemistry staining	Enhance of escape latencies in 5 μg/kg MC-LR-treated rats. Decrease of frequencies entering the enlarged platform in 1 and 5 μg/kg MC-LR-treated rats. No significant differences in the number of damaged neurons. Enhance of astrocyte density and NO concentration in hippocampus exposed to 5.0 μg/kg	Li et al. (2014)
Pure MC-LR	Rats	1, 5 or 20 μg/kg every 2 days for 8 weeks, by intragastric route. Later the rats became pregnant of a non-exposed male	Maternal toxicity and reproductive outcome, simple motor and locomotor activities, behavioral study and oxidative stress parameters	Decrease of mean body weight gain in maternal rats. Decrease of number of pregnant rats. Alteration of behavior and neurodevelopment in rat offsprings. Enhance of MDA and SOD in hippocampus of offsprings	Li et al. (2015b)
Extracted and purified MC-LR from blooms	Pregnant rats and pups	10 μg/kg daily from day 8 to postnatal day 15	Oxidative stress parameters, determination of MC-LR, histopathological study and protein expression	Enhance of MDA, Decrease of GSH and AChE activity. No significant PPs changes. 3.75 ± 0.94 ng/g d.w. were detected in brain of pup rats. Morphological changes. Alteration of proteins involved in neuronal processes in pup rats	Zhao et al. (2015)
Extracted and purified MC-LR from blooms	Mice	1 μL containing 1-20 ng/μL, by i.c.v. route. All parameters measured 3 h, 1 day, 3 day and 7 day after exposure	Behavioral study, histopathological study and oxidative stress parameters	Decrease of memory impairment. Morphological changes in hippocampal neurons from 10 ng/μL. Enhance of protein oxidation, LPO, ROS, SOD, GPx and Nrf2. Decrease of GSH/GSSG	Shin et al. (2018)
Pure MC-LR	Mice	1, 5, 10, 20 or 40 μg/L 12 weeks, by oral route	Histopathological study and protein expression	Pathological changes in hippocampus and cortical cells in a dose-dependent way. Differences between hippocampus and cerebral cortex in the affectation of mRNA and proteins expression: ATP6, COX3, CYTB, POLG, mtSSB and TFAM	Wang et al. (2018)
Pure MC-LR	Rats	3 μL of 0.1 μg MC-LR/μL (equivalent to 1.5 μg/kg) *via* hippocampal injection. All parameters measured 24 h, before and after exposure	Protein expression and behavioral study	Enhance of desmethylation of PP2Ac, phosphorylation of GSK-3β and tau, spatial memory deficit.	Zhang et al. (2018)

Abbreviations: AChE: acetylcholinesterase; ATP6: adenosine triphosphate-6; b.w.: body weight; CAT: catalase; COX3: cyclooxygenase-3; CYTB: Cytochrome B; EROD: cytochrome P-450-dependent 7- ethoxyresorufin O deethylase; GLU: glucose; GPx: glutathione peroxidase; GSH: reduced glutathione; GSK-3β: Glycogen synthase kinase 3 beta; GSSG: oxidized gluthatione; GST: Glutathione-S-transferase; i.c.v.: intracerebroventricular; LPO: lipid peroxidation; LTP: long term period; MDA: malondialdehyde; mtSSB: mitochondrial single-stranded DNA binding protein; NO: nitric oxide; POLG: DNA polymerase g; PP: protein phosphatase; PP2Ac: catalytic subunit of protein phosphatase 2A; ROS: reactive oxygen species; SOD: superoxide dismutase; TBARS: total thiobarbituric acid reactive species; TFAM: mitochondrial transcription factor A.

lighting the fact that AWC and AWA neurons act as independent targets. Moreover, these effects were compared to the ones caused by the exposure to MC-LF, suggesting a more potent effect by MC-LF than MC-LR. Up to date, only this neurotoxicity study has been carried out with this congener in nematodes, despite MC-LF is transported more efficiently into the neurons (Feurstein et al., 2010). Furthermore, Saul et al. (2014) obtained a significant decrease in all life trait variables, measured at different periods of the nematode life cycle, only at the highest concentration of MC-LR assayed. They investigated widely the variation in the gene expression, reporting an enhancement of 125, among which was unc-30, related to the GABAergic response, and a decrease of 76. Although these results may seem contradictory to the ones obtained by Ju et al. (2013), as they described a diminish of unc-30 gene expression, it is important to highlight that the duration of the stress exposure is essential for their regulation, being the possible cause for their discordance (Nadal et al., 2011). Moreover, Saul et al. (2014) also reported a down-regulation in *let-7* expression, which could play a role in the development and

the reproductive processes, contributing, therefore, to the effects observed in the brood size and growth. Their results manifested that many of the affected genes by MC-LR are involved in neurogenesis, signaling or neurological behavior processes, reinforcing those results previously obtained by Li et al. (2009a) and Ju et al. (2013), where MC-LR played an important role in the neuromodulating action.

In general, the different behavioral studies agree that MC-LR produced a decrease in autonomic (body bend, head thrash, move length, pharyngeal pumping, touch response) and sensory (chemical, thermal) functions reflecting an alteration in the nervous system functions to generate appropriate behaviors from sensory signals in nematodes (Li et al., 2009a, 2009b; Ju et al., 2013, 2014; Moore et al., 2014). Therefore, MC-LR at environmentally relevant concentrations, could affect the nervous system regulation to receive, process, integrate and interpret sensory signals, as suggested by the gene expression results (Li et al., 2009a, 2009b; Ju et al., 2013; Saul et al., 2014; Hu et al., 2016). It is important to point out that not always a variation in the gene expression can be translated to a change in protein levels, being required complementary studies in order to assure the neurotoxic role of this toxin (Saul et al., 2014). Although previous studies confirmed the suitability of the *C. elegans* test as a neurotoxicity screening test for MCs (Ju et al., 2014), it should be taken into account that this experimental model is much simpler than the mammals-nervous system.

The only neurotoxicity study performed in birds was carried out in Japanese quail exposed to *Microcystis* biomass containing MC-LR, MC-RR, MC-YR and MCs-similar compounds (Pašková et al., 2008). This study focused on the determination of oxidative stress, where a significant enhancement was reported in cytochrome P-450-dependent 7-ethoxyresorufin O-deethylase (EROD) levels in the brain after acute and sub-chronic exposure at medium concentrations of MCs. The LPO levels were also enhanced after acute and sub-chronic exposure, so did the GSH levels, decreasing, nonetheless, after acute exposure. Howbeit, no significant changes were observed in GST activity in this organ. In general, a rise in the oxidative stress parameters was described by these authors in brain (Pašková et al., 2008). Oxidative stress as a mechanism of toxic action of MCs has been widely studied in other organs such as liver or kidney in different species (Li et al., 2003; Jos et al., 2005; Skocovska et al., 2007; Weng et al., 2007; Prieto et al., 2009); however, these investigations are very scarce in brain. The increase of ROS could be involved in the mitochondrial dysfunction and activation of calpain and Ca^{2+}/calmodulin-dependent protein kinase II (Ding and Nam, 2003), generating damage in the brain structure and neurological functions.

Mice exposed to pure MC-LR showed differences in the effects on hippocampus and cortex after oral exposure by drinking water with 1–40 μg/L MC-LR for a year (Wang et al., 2018). Histopathological changes were observed in the hippocampus (bright eosinophil-like angular shape and nuclear fragments) and in the cortex (shrunken bodies and pyknotic nuclei) dose-dependently. Likewise, MC-LR produced different impacts on mRNA transcription genes and in their protein expression (ATP6, COX3, CYTB, DNA polymerase γ (POLG), mitochondrial single-stranded DNA-binding protein (mtSSB) and mitochondrial transcription factor A (TFAM)), mainly affecting the hippocampus. In accordance with these results, Shin et al. (2018) described a dose-dependent neuronal loss in the same hippocampal cells due to several morphological changes, but in this case, after exposure to a cyanobacterial extract containing MC-LR. Moreover, several behavioral studies demonstrated memory impairment after Morris water maze (MWM) and passive avoidance tests. However, these effects were only observed after exposure to 4 μg/mL

MC-LR, suggesting that the neuronal loss is not the main cause for these toxic effects in mice. After exposure to the same doses, no effects on spatial working and visual recognition memory were detected by Y-maze and novel object recognition tests, respectively. These effects were patent only in non-transgenic (non-Tg) mice compared to those overexpressing glutathione peroxidase (GPx Tg). Besides, in non-Tg group, these authors observed significant changes in oxidative stress biomarkers such as increased protein oxidation, LPO and ROS, together with a decrease of the GSH/Glutathione disulfide (GSSG) ratio. Moreover, the rise in SOD enzyme activity was more evident in non-Tg compared to the increase observed in GPx-1 Tg, while the enhancement of GPx enzyme activity was more visible in this last group of mice. Furthermore, no proinflammatory tumor necrosis factor-α (TNFα) and allograft inflammatory factor-1 (Iba1) levels were affected after the MC-LR exposure. All of the results obtained in this study suggest that memory impairments in mice exposed were due to oxidative stress in spite of by neuroinflammation process, which could be confirmed by the enhancement of nuclear factor erythroid-derived 2 (Nrf2) observed in both exposed groups. In addition, the reduced responses of GPx-1 Tg compared to non-Tg mice suggest a possible prevention of memory impairment by compounds implied in antioxidant activity (Shin et al., 2018).

Furthermore, it is important to highlight that, although not being neurotoxicological studies *per sé*, some studies exposing mice to pure MC-LR have proven its capacity to cause effects in the HPI axis at hypothalamic level, altering the neurohormonal control of reproduction (Wang et al., 2012; Xiong et al., 2014; Chen et al., 2016).

In rats exposed to MC-LR after infusion into hippocampus presented longer periods of time searching the platform and shorter swimming distance in the target zone, but no significant differences in swimming speed were appreciated compared to the control group (Li et al., 2012a). This would point out the spatial learning and memory impairment caused by the exposure to the toxin. Furthermore, some neuronal injury was observed by shrunk nuclei and cellular edema or dissolved cell organelles, diminishing significantly the number of CA1 pyramidal cells in the hippocampus. Nonetheless, after exposure to the lowest dose, some morphological changes were appreciated in the neurons, like swollen and degranulated endoplasmic reticulum or puffed periplast. In fact, a more significant rise in oxidative stress parameters was reported after exposure to the highest concentration (LPO, CAT, GPx, and SOD) *versus* the lowest (LPO and CAT). In agreement with these results, Li et al. (2012b) reported that chronic exposure produced neither changes in intake, body weight and overall mobility, nor visual and locomotor deficits, although they demonstrated the presence of this toxin in brain. However, treated rats did take longer to find the platform, mainly over the late days, spending less time in the target zone, which implies effects on spatial learning and memory as well. This impairment was also confirmed by the degeneration and apoptosis of hippocampal cells in rats exposed to MC-LR for 50 days. Furthermore, the authors observed the presence of proteins involved in neurodegenerative diseases such as septin 5, a-internexin and a-synuclein, and a PPs inhibition after exposure to 10 μg/kg MC-LR, which may lead to Tau hyperphosphorylation, implied in the generation of Alzheimer's disease. This is the first scientific study correlating MC-LR exposure to an age-associated neurodegenerative disorder.

Additionally, Wang et al. (2013) reported a significant PPs activity enhancement after exposure to pure MC-LR in rats, in disagreement with the results obtained by Li et al. (2012b). This effect could be the cause for the reduction, at all concentrations, of the phosphorylation of GSK-3β in the hippocampus and, consequently, for the described long-term potential concentration-dependent effects, leading to a loss

of neuronal plasticity. Moreover, this study showed, for the first time, the prevention of the neurotoxic effects caused by MC-LR by simultaneous treatment with a GSK-3β inhibitor.

In agreement with Li et al. (2012a, 2012b), an investigation of the effects of MC-LR on learning and memory ability in rats was performed by Li et al. (2014), obtaining that the rats exposed to the highest dose presented prolonged escape latencies on the third day of training, while those exposed to lower doses had shorter frequencies entering the enlarged platform. Despite no significant differences in the number of damaged neurons were observed, an increase of astrocyte cells density in the hippocampus was reported after the exposure to the highest dose. This could be related to the increase of nitrogen reactive species, an inflammatory indicator, reported in the hippocampus at the same dose, playing a role in the central neuron system inflammatory reactions and affecting spatial memory impairment.

The only study evaluating the transmission of the toxic effects of MC-LR in female rats to offspring was performed by Li et al. (2015b). In maternal rats, a decrease in the mean body weight gain was significant only at the highest dose of exposure. Respecting the behavior of the offspring, a significant reduction of the ability in the cliff avoidance test was observed, although no differences were perceived after the surface righting reflex and the negative geotaxis tests. However, no significant alterations in the locomotor activity were observed. In the MWM test, the frequencies in reaching the platform zone decreased dose-dependently in male offspring at all exposure doses, while in the case of female offspring, the diminishment of frequency was produced only after exposure to the highest doses, together with the effects on the swimming speed. Furthermore, although no evident pathological alterations in the hippocampus were observed, a significant increase of LPO and SOD levels were reported in male and female offspring after exposure to the highest dose, and an increase of LPO levels after 5 µg/kg MC-LR exposures only in male subjects.

Recently, Zhang et al. (2018) indicated an accumulation of MC-LR in the hippocampus after 24 h of injection, causing demethylation of PP2Ac (inhibition of PP2Ac) and phosphorylation of GSK-3β (activation of GSK-3β). This could lead to the hyperphosphorylation of Tau, being in agreement with Li et al. (2012b) and Wang et al. (2013). These results confirm the effects obtained, as mentioned above, in the SH-SY5Y *in vitro* model in the same study (Zhang et al., 2018). Moreover, going along with the results obtained in the MWM test by Li et al. (2012a, 2012b, 2014, 2015b), a reduction of the swimming distance spent in the target zone was observed as well, in this case, after day 8 compared to day 6, producing, consequently, memory impairments.

Although they represent a more realistic scenario, only two neurotoxicity studies have been carried out with MCs contained in extracts of cyanoblooms. In this sense, Maidana et al. (2006) used the step-down inhibitory avoidance test by injection of MC-LR containing raw extract. They reported a significant effect on long-term memory and the impairment of its retrieval at both doses assayed, while no significant changes were produced in short-term memory at any dose. Furthermore, using the radial arm maze to test the spatial memory, the number of working and reference memory errors increased only at day 8 of exposure at both concentrations, being probably caused by the accumulation of previous extracts-administrations. Surprisingly, an increase of the time spent to consume all the baits was reported in the same test only at the lowest dose. Moreover, these authors also studied different oxidative stress parameters, obtaining higher GST activity after exposure to the lowest dose compared to the highest. In the case of LPO levels, higher levels were obtained after exposure to

the highest MCs dose, although the lower dose also caused lipid peroxidation. These parameters could be the cause for the increase of DNA damage observed after exposure to MCs in the comet assay, corroborating the role of oxidative stress in the neurotoxic effects produced by MC-containing extracts, as was previously demonstrated with pure MCs (Li et al., 2012a, 2015b). In agreement with these oxidative stress results, Zhao et al. (2015a) obtained an increase in the LPO levels in the brain of the pups after maternal exposure to MCs-extract, together with a decrease in the GSH levels and in AChE activity in the cerebral cortex. Moreover, although these authors verified the presence of MC-LR in the offspring brains, no changes were obtained in the PP activity after maternal exposure, which would be in disagreement with the PP activity enhancement reported by Wang et al. (2013) and its decrease reported by Li et al. (2012b) and Zhang et al. (2018). This could be due to the experimental subjects since in both cases the parameter was measured in a direct object, the adult rats, *versus* an indirect object, their pups; or the discordance in the administration route, being, in this case, subcutaneous. Furthermore, similar ultrastructural changes were obtained in brain offspring by Li et al. (2012a). Likewise, an alteration of proteins involved in neurodevelopment was detected as well, in agreement with Li et al. (2012b).

Taken together, all the experiments conclude that MCs both pure and contained in cyanoblooms extracts produced important neurotoxic effects in several species by different exposure routes. Mostly, MCs caused oxidative stress and alteration of biochemical chains that ended up leading to huge effects such as hyperphosphorylation of Tau. In fact, most of them demonstrate the spatial learning and memory impairment by several behavioral tests. Howbeit, very few studies have been performed using other MC congeners besides MC-LR, isolated and contained in the mixture in a cyanobacterial extract, although some of them have demonstrated to exert more severe neurotoxic effects, being the case of MC-LF for instance.

3. Cylindrospermopsin

Cylindrospermopsin consists of a tricyclic guanidine group combined with a hydroxylmethyl uracil group (Ohtani et al., 1992). Its structure presents a zwitterionic nature and a low molecular weight (415 Da) (Falconer and Humpage, 2006). This cyanotoxin is produced by several cyanobacterial genera such as *Cylindrospermopsis, Aphanizomenon, Umezakia, Chrysosporum,* and *Anabaena,* among others (Harada et al., 1994; Banker et al., 1997; Shaw et al., 1999; Schembri et al., 2001) (Fig. 3). (Fig. 4.)
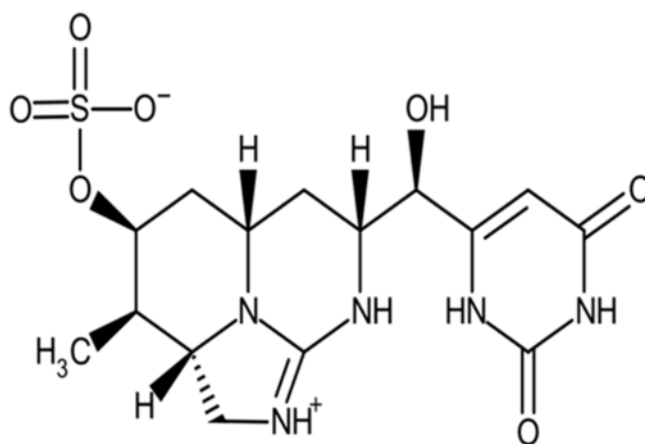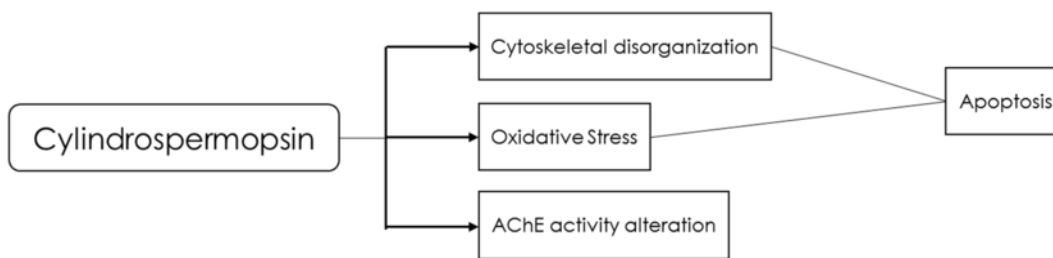

Fig. 3. Structure of CYN.

Fig. 4. Main mechanisms of neurotoxic action of CYN.

Despite being the liver its main target, many other organs such as kidneys, lungs, thymus, marrow bone, adrenal gland, gastrointestinal tract, immune and nervous systems, and heart have been described as potential targets as well (Hawkins et al., 1985; Terao et al., 1994; Falconer et al., 1999; Humpage et al., 2000; Guzmán-Guillén et al., 2015). The most well-known mechanism of action for CYN is the protein and GSH synthesis-inhibition (Terao et al., 1994; Runnegar et al., 1995; Froscio et al., 2003). In addition, due to its ability to enhance ROS production, this toxin can lead to DNA damage, causing cell death by apoptosis (Roos and Kaina, 2006; Gutiérrez-Praena et al., 2011; Puerto et al., 2011; Gutiérrez-Praena et al., 2012; Guzmán-Guillén et al., 2013) (Fig. 4). Moreover, some studies have demonstrated the importance of its previous metabolic activation by the enzymatic complex cytochrome P-450, since it is able to exert genotoxic potential (Runnegar et al., 1995; Norris et al., 2001; Froscio et al., 2003; Humpage et al., 2005; Žegura et al., 2011; Puerto et al., 2018). As a cytotoxin, these effects could be also caused in the nervous system. Besides, it is important to notice that the chemical structure of CYN is more alike to neurotoxins than to hepatotoxins, as it was classified at first, not being unexpected for this cyanotoxin to also cause neurological disorders (Kiss et al., 2002). Furthermore, although it is not likely for CYN to cross the BBB by passive diffusion due to its hydrophilic properties (Banks, 2009), its low molecular weight might play a role in its entrance to the nervous system. There are some studies pointing out its neurotoxicity in different *in vitro* and *in vivo* models, although the mechanisms for which CYN could exert neurotoxic effects in the brain remain unknown.

3.1. Neurotoxicological in vitro studies performed with cylindrospermopsin

Up to date, in comparison with MCs, very few studies have brought to light the potential neurotoxicity CYN can exert (Table 5). Furthermore, most of them have been performed using extracts or cultures of *Cylindrospermopsis raciborskii* or *Aphanizomenon ovalisporum*. In this sense, the first study suggesting its neurotoxic effect was performed by Kiss et al. (2002), who exposed CNS neurons of two species of snail, *Helix pomatia* L. and *Lymnaea stagnalis* L., to a *C. raciborskii* purified fraction and ATX-a. They suggested that the purified fraction could be CYN, and although it had no direct effect on the membrane of the neurons, it decreased the ACh-induced membrane response, suggesting a neuroactive effect on the cell membrane for the first time. On the contrary, Vehovszky et al. (2013) reported that application of a CYN-producing strain to CNS preparations of *H. pomatia* (at 20 mg/mL) did not display the same cholinergic inhibitory effects, although these were observed after exposure to a non-CYN-producing *C. raciborskii* bloom, which authors attribute to some ATX-a like compound.

In the case of CYN, contrary to MCs, there is only one work with pure toxin, performed by Takser et al. (2016). These authors evaluated *in vitro* the individual and combined effects of CYN, MC-LR

and ATX-a, at environmentally relevant low concentrations (10 μM alone and 3.3 μM in mixture), in brain cell lines. Their findings revealed that CYN individually and the mixture containing CYN were 3–15 times more potent than the individual toxins, inducing apoptosis and inflammation in murine BV-2 microglia cells and N2a murine neuroblasts cells. Besides, the latest were more sensitive to the mixture than BV-2 cells, causing a meaningful pro-inflammatory response to CYN and the mixture, demonstrating that low concentrations of CYN are highly relevant for neurodegeneration. These outcomes could have potential implications in future research on neurodegenerative diseases. Nevertheless, care should be taken in the extrapolation of these *in vitro* results to *in vivo* circumstances, including human health effects, mainly concerning the developing brain where there is no BBB yet.

3.2. Neurotoxicological in vivo studies performed with cylindrospermopsin

Studies concerning CYN neurotoxicity *in vivo* are scarce (Table 5), although they provide interesting results. In this regard, White et al. (2007) reported that 7 day-exposure of *Bufo marinus* tadpoles to whole cell extracts or live cultures of *C. raciborskii* at 400 or 232 μg/L, respectively, appeared to decrease their activity levels, mostly swimming behavior, which could make them more vulnerable to prey, but also be used as an avoidance strategy from visually-oriented hunters. This effect, however, might have been caused by damage in some other organs. It is worth to mention that live *C. raciborskii* cultures contained a mixture of intra- and extracellular CYN, whereas the cell extracts only had extracellular CYN, and they also reported the presence of deoxy-CYN. This work by White et al. (2007) was the first one using amphibians as experimental model, whose changes in behavior gain relevance as they are usually the first indication of sublethal exposure (Henry, 2000), being a possible indicator of CYN neurotoxicity. In agreement with these results, Kinnear et al. (2007), using the same model and conditions, but nearly half the concentrations (200 and 107 μg/L, for the cell extracts and the live cultures, respectively), reported a reduction in the swimming ability and un-coordination in tadpoles of *B. marinus*. They suggested that it could be due to the disintegration of the brain, as the encephalon had a loosely arranged matrix and brain cells were disintegrated and sometimes necrotic, showing a mix of the outer matrix and inner cells, together with general organ failure. Besides, authors also hypothesized that degeneration of the gill epithelia could have led to suffocation, and finally to the consequently reduced activity.

To our knowledge, there are only two studies concerning the neurotoxicity of CYN in fish. CYN was detected by ELISA in the brain of all tilapia fish (*Oreochromis niloticus*) exposed subchronically (14 days) by immersion to repeated concentrations (10 μg/L) of an *A. ovalisporum* culture containing CYN and deoxy-CYN (Guzmán-Guillén et al., 2015). As a result, a marked increase in LPO levels, and a reduction in AChE activity in tilapia brains was observed, al-

Table 5
Neurotoxicity studies performed with CYN.

CYN/Cyanobacteria	Experimental model	Experimental conditions	Assays performed	Relevant Results	References
In vitro					
Crude extracts of *C. raciborskii*	Neurons of *Helix pomatia*	Extracts of both the bloom sample and the laboratory isolate of the bloom were diluted in physiological *Helix* saline and applied by perfusion at a constant flow rate	Electrophysiological experiments	No cholinergic alteration was observed with the CYN-producing strain	Vehovszky et al. (2013)
Pure CYN	N2a murine neuroblastoma derived cells	0.001, 0.1 and 10 μM for 24, 48 and 72 h	MTT assay Apoptotic cell death TNF-α measurement	Concentration and time-dependent decrease of cell viability after all time exposures to both 0.1 and 10 μM. Significant rise in proapoptotic caspases after exposure to 10 μM	Takser et al. (2016)
	BV-2 microglia murine cells			Concentration-dependent decrease of cell viability after all exposure times to both 0.1 and 10 μM. Significant rise in proapoptotic caspases after exposure to 10 μM	
In vivo					
Whole cell extracts of *C. raciborskii* and live cultures of *C. raciborskii*	*Bufo marinus* tadpoles	0–200 and 0-107 μg/L, respectively, for 7 days, by transdermal route	Histopathological study	No mortality observed. Several histopathological changes in the encephalon	Kinnear et al. (2007)
Whole cell extracts of *C. raciborskii*	*Bufo marinus* tadpoles	0–400 μg/L for 7 days, by transdermal route	Behavioral studies Toxin analysis	Decrease in behavior scores Neither mortality nor growth rates were affected	White et al. (2007)
Live cultures of *C. raciborskii*		0–232 μg/L for 7 days, by transdermal route		Decrease in behavior scores Time-dependent increase in mortality Negative growth rates	
A. ovalisporum culture containing CYN	Tilapia fish (*Oreochromis nicotilus*)	10 μg/L for 14 days, by transdermal and oral route	AChE activity, LPO, histopathological study and ELISA	Inhibition of the AChE activity Rise in LPO levels Necrosis, hyperemia, haemorrhagia and edema CYN detection in all brain samples	Guzmán-Guillén et al. (2015)
Purified CYN (CYNp) and extract of *C. raciborskii* containing CYN (CYNex)	Trahira (*Hoplias malabaricus*)	Single dose of 50 μg/kg b.w. for 7 and 14 days by intraperitoneal injection	AChE activity, GST activity, LPO and ELISA	Increase of AChE activity after 7 days of exposure to CYNex, decreasing after 14 days. Decrease of GST after 7 days of exposure to CYNex and increase after 7 days of exposure to CYNex and after 14 days of exposure to CYNp and CYNex. Rise in LPO levels after 7 and 14 days of exposure to CYNp and CYNex. Detection of CYN in brain	da Silva et al. (2018)

Abbreviations: *A. ovalisporum*: *Aphanizomenon ovalisporum*; AChE: Acetylcholinesterase; BV-2: cellosaurus cell line; b.w.: body weight; *C. raciborskii*: *Cylindrospermopsis raciborskii*; CYN: cylindrospermopsin; CYNp: purified cylindrospermopsin; CYNex: extract containing cylindrospermopsin; GST: glutathione-S-transferase; LPO: lipid peroxidation; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N2a: fast-growing mouse neuroblastoma cells.

though the inhibition of AChE activity was too low to induce neurological symptoms. In addition, signs of necrosis, vacuolization, chromatin condensation, cytoplasmic edema and mitochondrial swelling were reported as well. Recently, detection of CYN has also been reported in brains of the fish *Hoplias malabaricus* exposed by a single i.p. injection (50 μg/kg b.w.) to purified CYN or to extracts of a CYN-producing strain of *C. raciborskii*, even 7 and 14 days after exposure (Da Silva et al., 2018). In addition, detected CYN levels were higher after exposure to the extracts, which could point out the importance of other compounds in the extract (*i.e.* lipopolysaccharides) that might also affect CYN crossing of the BBB. Nonetheless, no significant effects were noticed on AChE activity after CYN exposure in any form tested, contrary to the results obtained by Guzmán-Guillén et al. (2015), which could be due to differences in the exposure concentrations and times (subchronic *versus* acute exposure) or in the fish species, although both studies agree on the rise of LPO levels. Moreover, GSH levels did not vary in *H. malabaricus* after exposure to CYN, but different responses were obtained for GST activity for extracts and pure CYN. To exert neurotoxic effects, toxins must be transported into cells or interact with channels or receptors of the cell membrane (Stillwell, 2013), suggesting the interference of other compounds present in the extract (Da Silva et al., 2018).

Some neurological symptoms after exposure of alligators (Schoeb et al., 2002) and mice (Saker et al., 2003; Zagatto et al., 2012) to *C. raciborskii* strains have been attributed to CYN (Poniedziałek et al., 2012). However, it is important to clarify that neither of these studies proved the presence of CYN in those strains, so the reported effects might be due to different compounds present in the extracts or different secondary metabolites, such as STX.

4. Conclusions

This review summarizes, as far as we know, the reports available on the scientific literature dealing with the neurotoxicity assays performed *in vitro* and *in vivo* to elucidate the toxic effects that MCs and CYN can exert in the nervous system. In the case of MCs, they have proven to cause neurotoxicity by their crossing using the OATPs, which are present in the BBB and in most neural cells, leading to a rise in the $[Ca^{2+}]_i$ levels and, therefore, apoptosis. These cyanotoxins have demonstrated to exert neurotoxic effects mostly in the limbic system. In fact, some histopathological studies have described impor-

tant damages in the hippocampus and in the cortex, together with global biochemical alterations, being especially relevant Tau hyperphosphorylation, characteristic of some neurodegenerative diseases such as Alzheimer's disease. On the other hand, these toxins have proven to cause damage in the hypothalamus as well, having an impact in other systems of the organism such as the reproductive or the endocrine. Furthermore, MCs have exerted a rise in oxidative stress and lipid peroxidation, together with neurotransmission alterations (DA, ACh and GABA levels), leading to autonomic and sensory responses. Thus, MCs not only cause effects in the CNS but also in the peripheral nervous system. Furthermore, some other minor variants such as MC-LF or MC-LW require attention as well, since both have demonstrated to be even more toxic in neural cells, in spite of being less environmentally abundant. Special attention should be paid to the fact that very little studies have been carried out *in vivo* using one of the major congeners in nature, MC-RR. In the case of CYN, the number of studies performed is even scarcer, reporting deregulation of some oxidative stress parameters was observed together with alteration of AChE activity, which could be linked to the histological changes observed. Thus, although neurotoxicity mechanisms for CYN are still unknown, it seems to be caused by damage in the CNS. For all mentioned above, further research is required in order to clarify the neurotoxic potential of several MC congeners and CYN, as well as their possible contribution in neurodegenerative diseases.

Author contributions

All authors have contributed by search, selecting, processing, writing and reviewing the information collected in this review.

Uncited reference

Okuzawa et al., 2003

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CHAPTER 3

María Gracia Hinojosa, Ana Isabel Prieto, Daniel Gutiérrez Praena,

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IN VITRO ASSESSMENT OF THE COMBINATION OF

CYLINDROSPERMOPSIN AND THE ORGANOPHOSPHATE

CHLORPYRIFOS ON THE HUMAN NEUROBLASTOMA SH-SY5Y

CELL LINE

1   ***In vitro*** **assessment of the combination of** ~~cylindrospermopsin~~**Cylindrospermopsin**

2   **and the organophosphate chlorpyrifos on the human neuroblastoma SH-SY5Y cell**

3   **line**

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Abstract

31   Cylindrospermopsin (CYN) is a cyanotoxin which occurrence is increasing due

32   to climate change. This toxin is able to exert damage in the organism at several levels,

33   among them, inwhich is the nervous system. Moreover, it is important to take into

34   account that this toxin is not usually present isolated in nature, but in combination with

35   some other pollutants, being the case of the pesticide chlorpyrifos (CPF). Thus, the aim

36   of the present work was to assess the effects of the interaction of CYN in combination

37   with CPF in the human neuroblastoma cell line SH-SY5Y by evaluating cytotoxicity

38   and mechanistic endpoints. The mixtures 0.25+21, 0.5+42, 1+84 µg/mL of CYN+CPF,

39   based on cytotoxicity results, were evaluated, and the isobologram method detected an

40   antagonistic effect after 24 and 48 hours of exposure. Moreover, although no alterations

41   of reactive oxygen species were detected, a significant decrease of glutathione levels

42   was observed after exposure to both, CPF alone and the combination, at all the

43   concentrations and times of exposure assayed. In addition, CYN + CPF caused a

44   marked decrease in the acetylcholinesterase activity, providing similar values to CPF

45   alone. However, these effects were less severe than expected. All these findings,

46   together with the morphological study results obtained, point out that it is important to

47   take into account the interaction of CYN with other pollutants. Further research is

48   required to contribute to the risk assessment of CYN and other contaminants

49   considering more realistic exposure scenarios.

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Keywords: ~~cylindrospermopsin~~Cylindrospermopsin, chlorpyrifos, SH-SY5Y cells, chemicals combination, pollutants

1. Introduction

Cyanobacterial blooms are a global environmental concern due to their increased occurrence in terrestrial, marine and freshwater ecosystems, caused by climate change and human influence. Among their detrimental effects, they produce unpleasant organoleptic properties where they proliferate (Manning and Nobles, 2017). However,~~But~~ the main hazard is their capacity to produce cyanotoxins, toxic secondary metabolites that threaten water safety and aquatic life (Brooks et al., 2017; Buratti et al., 2017). There are different exposure routes to them~~cyanotoxins~~, standing out the oral one as one of the most common and dangerous.~~, by the intake of contaminated water or food (Gutiérrez-Praena et al., 2013).~~ Nonetheless, other relevant routes such as dermal contact or inhalation are worth to be mentioned as well (Buratti et al., 2017).

Cylindrospermopsin (CYN) is one of the most common aquatic cyanotoxins as a consequence of the increasing occurrence of *Cylindrospermopsis raciborskii* blooms, the most relevant producer species (Antunes et al., 2015). However, other cyanobacterial species such as *Chrysosporum ovalisporum, Aphanizomenon flos-aquae, Anabaena bergii,* or *Raphidiopsis curvata* are able to produce this toxin as well (Manning and Nobles, 2017). Cylindrospermopsin consists of an alkaloid with a tricyclic guanidine linked to a hydroxy~~l~~methyl uracil group (Ohtani et al., 1992). The main episode~~case~~ of human intoxication by CYN occurred in the outbreak of 1979 in Palm Island (Australia)~~,~~ when 146 people were hospitalized in a local clinic with malaise, vomits, anorexia and hepatomegaly after drinking from a water supply

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containing CYN-producing *C. raciborskii* (Bourke et al., 1983; Griffiths and Saker, 2003). However, despite intoxication by drinking contaminated water, it is also feasible to be exposed by ingestion of contaminated food. In fact, the bioaccumulation of these cyanotoxins by plants irrigated with contaminated water (Testai et al., 2016) or in exposed aquatic organisms has been demonstrated, enhancing the risk of human intoxication by the food chain (Cordeiro-Araújo et al., 2017; Gutiérrez-Praena et al., 2013; Machado et al., 2017).

Although its target organ is the liver, CYN has widely demonstrated its capacity to damage other organs, including the nervous system (Guzmán-Guillén et al., 2015; Falconer, 1999; Hawkins et al., 1985; Humpage et al., 2005; Terao et al., 1994). Its main mechanism of action is the irreversible inhibition of protein and glutathione (GSH) synthesis (Froscio et al., 2003). Moreover, CYN has demonstrated its ability to increase reactive oxygen species (ROS) production, linked to apoptosis and DNA damage (Guzmán-Guillén et al., 2013; Puerto et al., 2011), and progenotoxic effects in several cell lines (Puerto et al., 2018; Žegura et al., 2011). In addition, several studies have shown the capacity of CYN to cause neuronal damage *in vitro* (Hinojosa et al., 2019a; Takser et al., 2016) and *in vivo* (da Silva et al., 2018; Guzmán-Guillén et al., 2015), by inducing changes in the acetylcholinesterase activity (AChE) and oxidative stress (Hinojosa et al., 2019b).

Furthermore, the pollution of water by pesticides and their metabolites has been of great concern due to the increasing number of pesticides used in agricultural practices and detected in water, as well to their persistence, mobility, and toxicity (Carvalho, 2012). Among all types of pesticides, organophosphates (OP) are about 38% of the global pesticides used, due to their high effectiveness against target pests and relatively low toxicity to non-target organisms (Koly and Khan, 2018). One of the most common

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OP used in agriculture and residential pest is chlorpyrifos (CPF) [O0, O0-diethyl 0-(3,

5, 6- trichloro-2-pyridinol) phosphorothionate] (Mehta et al., 2009). This OP has been

detected in many samples from all over the world (Koly and Khan, 2018).

The cases of human intoxication with CPF are numerous, including symptoms of

headache, dizziness, nausea, sweating, salivation, muscle twitching, unconsciousness,

convulsion, and death (Eaton et al., 2008). The main mechanism of action for CPF is the

inhibition of the acetylcholinesterase activity, which leads to accumulation of

acetylcholine in the synaptic space, causing an excessive stimulation of postsynaptic

neuronal receptors and consequent signs of toxicity (Al-Badrany and Mohammad, 2007;

Mehta et al., 2009; Zheng et al., 2000). Moreover, CPF induces changes on

macromolecule synthesis (DNA, RNA, proteins), on neurotransmitter receptors, and in

signal transduction pathways, neuronal differentiation, and neurochemical effects.

Furthermore, it is also capable of producing oxidative stress by lipid peroxidation and

increasing ROS (Uchendu et al., 2012).

Recently, the European Food Safety Authority has established the need of

studying the toxicity of the mixtures of cyanotoxins and other chemicals, as some OP

pesticides were reported to potentiate the anatoxin-induced toxicity, for instance (Cook

et al., 1988, Testai et al., 2016). In this sense, due to the possible coexistence of CYN

and CPF in water or theirby bioaccumulation in aquatic organisms or crops, the aim of

the present work was to study, for the first time, the potential interaction and effects of

the combination of the cyanotoxin CYN and the pesticide CPF in the human

neuroblastoma cell line SH-SY5Y, including cytotoxicity, oxidative stress, AChE

inhibition, and morphological changes.

2.   Materials & Methods

2.1. Supplies and chemicals

Cylindrospermopsin~~YN~~ (purity > 95% by HPLC) was purchased from Enzo Life Sciences. Nutrient Mixture F-12 Ham and CPF were purchased from Sigma-Aldrich (Madrid, Spain). Minimum essential medium (MEM), fetal bovine serum (FBS) and cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain).

The Bradford reagent was purchased from Sigma-Aldrich (Madrid, Spain). The supplier of MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt) Cell Titer 96® AQueous One Solution Cell Proliferation Assay was Promega (Biotech Iberica, Madrid, Spain).

2.2. Model system

SH-SY5Y cells, derived from a human neuroblastoma, were obtained from the American Type Culture Collection (CRL-2266). These cells were maintained in a MEM and F-12 (1:1) medium supplemented with 10% FBS, 1% L-glutamine 200 mM, 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin solution, in an atmosphere containing 5% CO_2 at 95% relative humidity at 37ºC (CO_2 incubator, NuAire®, Spain). Cells were grown 80% confluent in 75-cm^2 plastic flasks and harvested twice a week with 0.25% trypsin-EDTA (1X). The quantification of the cells was performed in a Neubauer chamber. SH-SY5Y cells were plated at density of $1 \cdot 10^5$ cells/mL to perform all the experiments.

2.3. Toxin test solutions

Stock solution of 1 mg/mL CYN was prepared in sterilized milliQ water, and maintained at -20˚C until its use. Stock solution of 50 mg/mL CPF was prepared in absolute ethanol and maintained at -20˚C until its use.

2.4. Cytotoxicity assays

149   SH-SY5Y cells were seeded for basal toxicity tests in 96-well tissue-culture

150   plates and, after being incubated at 37°C for 24 hours, the exposure took place. From

151   the CYN-stock solution, serial dilutions in medium without serum were prepared (0-1

152   µg/mL CYN) based on previous studies (Hinojosa et al., 2019a). From the stock

153   solution of CPF, serial dilutions in medium without serum were prepared (0-200 µg/mL

154   CPF). This concentration was selected based on previous studies (Amani et al., 2016;

155   Fu et al., 2019). Non-treated cells were used as negative control. A solvent control

156   was also evaluated to ensure its innocuous role (data not shown). After replacing the

157   medium, the addition of the exposure solutions to the plates and incubation for 24 and

158   48 hours at 37°C took place. The endpoints assayed were protein content (PC) and

159   tetrazolium salt reduction (MTS). All the assays were performed by triplicate. All the

160   endpoints assayed are well described in Hinojosa et al. (2019a).

161   *2.5. Assessment of the effect of CYN-CPF combination by the isobolograms method*

162   Concentrations used to evaluate the toxic potential of the combination CYN-

163   CPF were chosen based on the cytotoxicity results of CYN and CPF previously

164   obtained (Hinojosa et al., 2019a). The highest exposure concentrations for the

165   combination studies were those obtained as the mean effective concentration (EC_{50}) of

166   the most sensitive endpoint, in this case, the MTS assay at 24 hours, along with the

167   fractions $EC_{50}/2$ and $EC_{50}/4$. Thus, SH-SY5Y cells were exposed for 24 and 48 hours to

168   binary pure pollutants combinations: EC_{50} CYN + EC_{50} CPF, $EC_{50}/2$ CYN + $EC_{50}/2$

169   CPF and $EC_{50}/4$ CYN + $EC_{50}/4$ CPF.

170   The isobologram method was used according to Tatay et al. (2014) to determine

171   the type of interaction occurring when CYN and CPF are combined. This method

172   consists of plotting the concentration-effects curves for each compound and their

173   combinations in multiple diluted concentrations using the median-effect equation. The

174   combination index (CI) quantifies the synergism (CI < 1), the additivity (CI = 1), or the

175   antagonism (CI > 1) of the combinations. The CalcuSyn software calculates these CI

176   values automatically (Biosoft, Cambridge, UK).

177   *2.6. Oxidative stress assays*

178   *2.6.1. Reactive Oxygen Species (ROS) generation*

179   The ROS production was assessed in 96-well plates using the

180   dichlorofluorescein (DCF) assay, previously optimized by Medrano-Padial et al. (2019).

181   Fluorescence was measured at 535 nm (emission) and 485 nm (excitation) after

182   incubation for 4, 8, 12 and 24 hours.

183   *2.6.2. Glutathione (GSH) content*

184   The GSH content was measured according to Maisanaba et al. (2018). Cells

185   were exposed and incubated for 4, 8, 12 or 24 hours. Buthionine sulfoximine (BSO) 1μM

186   was used as positive control. Fluorescence was measured 460 nm (emission) and 380

187   nm (excitation).

188   *2.7. Acetylcholinesterase (AChE) activity determination*

189   Acetylcholinesterase activity was measured following the method of Ellman et

190   al. (1961) with modifications (Santillo et al., 2015). Viable SH-SY5Y cells were

191   exposed to the toxins and incubated at 37ºC for 24 hours. A solution of parathion 50 nM

192   was used as a positive control. Then, the medium was discarded and replaced by 200 μL

193   of a reaction mixture containing 0.5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB)

194   and 100 μM acetyltiocholine (ATCh) per well. The resulting product of the reaction, 5-

195   thio-2-nitrobenzoate (TNB), was measured at 410 nm every 90 seconds up to 1 hour.

196   *2.8. Morphology*

197   The concentrations used for the morphological study were the ones obtained in

198   the cytotoxicity assays, being the EC_{50} values those obtained after 24 hours of exposure.

199   These values were chosen as the highest concentrations of exposure along with the

200   fractions $EC_{50}/2$ and $EC_{50}/4$. SH-SY5Y cells were exposed for 24 hours. The procedures

201   for the contrast, light and electron microscopies were described by Hinojosa et al.

202   (2019a).

203   *2.10. Calculations and statistical analysis*

204   Data for the cytotoxicity assays, oxidative stress biomarkers and AChE were

205   presented as mean ± standard deviation (SD) ~~in relation~~ to control. Analysis of variance

206   (ANOVA) followed by Dunnett's multiple comparison was carried out to perform the

207   statistical analysis, using GraphPad InStat software (GraphPad Software Inc., La Jolla,

208   USA). Differences were considered significant from $P<0.05$. EC_{50} values were the

209   result of the linear regression in the concentration-response curves.

210   **3.   Results**

211   *3.1. Cytotoxicity assays*

212   A concentration-~~-~~dependent decrease in the viability was observed in the SH-

213   SY5Y cells after being exposed to 0-200 µg/mL CPF for 24 and 48 hours (Fig. 1). The

214   EC_{50} values obtained were 83.98 ± 2.74 µg/mL and 85.31 ± 7.67 µg/mL after 24 hours

215   of exposure for the MTS assay and the PC assay, respectively. In the case of the cells

216   exposed to CPF for 48 hours, the EC_{50} values were 69.78 ± 6.02 µg/mL and >200

217   µg/mL for the MTS and PC assays, respectively. Thus, the MTS assay demonstrated to

218   be the most sensitive biomarker.

219   *3.2. Assessment of the effect of CYN-CPF combination by the isobolograms method*

220   Using the EC_{50} value previously indicated in Hinojosa et al. (2019a), the

221   exposure concentrations of CYN were 0.25, 0.5 and 1 µg/mL, and ~~while for CPF were~~

222   21, 42 and 84 µg/mL of CPF, being both combined in a proportion of 1:84 for 24 or 48

223   hours (Fig. 2). The experiments performed with the combination presented a CI>1 in the

224   case of the cells exposed for 24 hours, confirming an antagonistic response (Fig. 3A).

225   However, in those cells exposed for 48 hours, the response obtained was mainly

226   synergic at low concentrations (CI<1) and antagonistic at higher ones (CI>1) (Fig. 3B).

227   *3.3. Oxidative stress assays*

228   The exposure to 21, 42 or 84 µg/mL CPF led to no significant changes in the

229   ROS levels compared to the control group in SH-SY5Y cells after any of the times of

230   exposure (4, 8, 12 or 24 hours) (Fig. 4A). However, it caused a significant decrease of

231   the GSH levels after all the times of exposure to the three concentrations assayed (Fig.

232   4B).

233   Similarly, the combination of the cyanotoxin and the ~~organophosphate~~ OP (1:84)

234   caused a reduction of the GSH levels in all the concentrations and times of exposure

235   assayed (Fig. 4D), despite the ROS levels were not altered compared to the control

236   group (Fig. 4C).

237   *3.4. Acetylcholinesterase activity*

238   All the concentrations of CPF (21, 42 and 84 µg/mL) reduced the AChE activity

239   in SH-SY5Y cells. Concerning the combination, all the concentrations assayed induced

240   a reduction of the AChE activity (Fig. 5).

241   *3.5. Morphology study*

242   Unexposed SH-SY5Y cells observed under phase-contrast microscop~~y~~e showed

243   a star-shape morphology with neurites contacting adjacent cells. The presence of mitotic

244 processes evidenced the active proliferation of these cells (Fig. 6A). The light

245 microscope revealed clear euchromatic nuclei with obvious nucleoli (Fig. 6B). Under

246 electronic microscope, cells showed a big number of mitochondria in the cytoplasm,

247 which also presented vacuole with a dense matrix (Fig. 6C).

3.5.1. Microscope observations of cells exposed to CPF

249 Cells exposed to 42.5 and 84 µg/mL CPF presented, under phase-contrast and

250 light microcopies, cell death signs. Cytoplasmic projections diminished with a low

251 proliferation rate. At the lowest concentration, a vacuolization of the cytoplasm was

252 observed (Fig. 6D-E). Ultrastructurally, mitochondrial organelles increased their size

253 due to the entrance of water. In addition, an alteration of the pattern of the

254 mitochondrial crests was observed. These cellular processes drove to the presence of

255 heterophagosomes and to condensation of the chromatin, a clear sign of apoptosis (Fig.

256 6F).

3.5.2. Microscope observations of cells exposed to the combination CYN+CPF

258 Concerning the combination, an intense vacuolization of the cytoplasm, cellular

259 cycle detention, loss of cytoplasmic projections, and cell death were observed at the

260 three concentrations assayed (Fig. 6G-H). Under the transmission electronic

261 microscope, small lipid droplets associated with mitochondrial organelles were

262 observed. Moreover, at the highest dose assayed (1 µg/mL CYN + 84 µg/mL CPF),

263 rough endoplasmic reticulum (RER) cisternae appeared dilated with dense content, and

264 an intense presence of heterophagosomes was observed (Fig. 6I).

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4. Discussion

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267   The interactive effects of chemicals and natural stressors have been reviewed

268   (Holmstrup et al., 2010; Laskowski et al., 2010). Synergistic interactions were reported

269   in more than 50% of the available studies of interactions between chemicals with abiotic

270   stressors. The coexistence of multiple cyanotoxins or with some other active

271   compounds has been described (Al-Sammak et al., 2014; Chen et al., 2017; Martínez-

272   Ruiz and Martínez-Jerónimo, 2016; Pathmalal, 2019; Tatters et al., 2017). However,

273   although pesticides are candidates to appear together with cyanotoxins, literature

274   regarding this subject is almost non-existent (Asselman et al., 2014; Daam et al., 2011;

275   Moe et al., 2012). In this sense, EFSA has highlighted the importance of addressing the

276   concomitant exposure of other chemicals, such as pesticides, along with cyanotoxins in

277   order to have information on mixture toxicity and on the environmental factors affecting

278   toxicity of a bloom (Testai et al., 2016).

279   The present work focused on the potential effects induced by the combination of

280   the cyanotoxin CYN and the organophosphate CPF in the neuronal SH-SY5Y cell line.

281   To address this research, a cytotoxicity study of CYN and CPF in the cell model was

282   required in order to establish the EC_{50} values of the test items to be applied in the

283   combination study. In the case of CYN, the EC_{50} value of 1 µg/mL CYN was taken

284   from a previous work, where SH-SY5Y cells were exposed to 0-10 µg/mL CYN for 24

285   and 48 hours (Hinojosa et al., 2019a). Regarding to CPF our study confirms its

286   damaging properties in neuronal cells, showing a time- and concentration-dependent

287   cytotoxicity. In agreement with our results, Park et al. (2013) demonstrated that CPF

288   induced cytotoxic effects in a concentration-dependent manner in SH-SY5Y cells

289   exposed to 0-70 µM CPF, establishing an EC_{50} value of ~32 µg/mL CPF after 24 hours

290   of exposure. Similarly, Raszewski et al. (2015) also found a time- and concentration-

291   dependent cytotoxic behavior of CPF in SH-SY5Y cells exposed during 24, 48, and 72

292   hours to 0-175 µg/mL CPF, obtaining an EC_{50} value for 24 hours ~~of ~~~of ~100 µg/mL

293   CPF.

294   The potential mechanisms by which CPF induces its neurotoxic effects are

295   oxidative stress and acetylcholinesterase inhibition, among others (Park et al., 2013;

296   2015). In our study, CPF did not show any alteration of ROS levels at any of the

297   concentrations assayed. However, GSH levels presented a concentration-dependent

298   decrease, which could indicate that GSH is scavenging ROS. In this sense, Park et al.

299   (2015) and Xu et al. (2017) found an increment in ROS levels when SH-SY5Y cells

300   were exposed to CPF. However, they did not evaluate the GSH levels, which could give

301   an idea about the antioxidant activity of the neuroblastoma cell line. On the other hand,

302   Giordano et al. (2007) evaluated GSH levels of SH-SY5Y cells exposed to CPF. The

303   authors did not find alterations in the tripeptide levels, although they observed and

304   increment in oxidized GSH levels, which indicated that an antioxidant response was

305   taking place, suggesting that cytotoxicity may be mediated by oxidative stress. In

306   addition, Park et al. (2013, 2015) suggested that CPF-oxidative stress induction was

307   related to~~with~~ AChE inhibition, together with its usual mechanism of action. In

308   agreement with this, CPF induced a clear inhibition of the AChE activity at all the

309   concentrations assayed in this case. Other authors have also pointed out that a depletion

310   of GSH levels is linked to ~~with a ~~mitochondrial dysfunction, which could lead to

311   apoptotic processes (de Oliveira et al., 2016; Park et al., 2013). This is in concordance

312   with our morphological findings, where the highest concentrations of CPF induced

313   mitochondrial alterations, leading to cell death by apoptosis. This process is related to

314   the activation of caspase-9 and -3 and an increment of cytosolic cytochrome c, as Park

315   et al. (2015) reported for SH-SY5Y cells exposed to CPF. Considering this, Raszewski

316   et al. (2015) proposed that CPF induces neurotoxicity through apoptotic mechanisms.

317   Once the toxic effects of CYN (Hinojosa et al., 2019a) and CPF alone on the

318   neuronal SH-SY5Y cell line were elucidated, the behavior of their combination on the

319   same cell line was evaluated. Our results showed that the combination was more

320   cytotoxic than both toxicants alone after 24 and 48 hours. However, in order to establish

321   the type of interaction between both compounds, the isobologram method was used.

322   Thus, after 24 hours of exposure, an antagonistic response was observed. This pattern

323   changed aAfter 48 hours, this pattern changed, showing a synergistic response at low

324   concentrations, and turning to an antagonistic response at higher concentrations. A

325   similar pattern was found when the combined toxicity of the cyanotoxin Microcystin

326   (MC)-LR and Cu exposure was investigated in the aquatic plant *Vallisneria natans*,

327   producing synergistic effects when combined at low concentrations (Wang et al., 2017).

328   Considering this, it is noteworthy to mention that CYN usually appears at low

329   concentrations in nature (de la Cruz et al., 2013), making a synergistic toxic response

330   very likely to appear.

331   To our knowledge, no studies concerning the effects of the combination of

332   cyanotoxins and pesticides using neuronal cell lines have been carried out. However, *in*

333   *vivo,* the first combination of cyanobacteria and pesticides was performed by Cerbin et

334   al. (2010), using a strain of *Microcystis aeruginosa* and the carbamate carbaryl. In this

335   study, the combination demonstrated a mostly additive interaction in *Daphnia pulicaria*

336   after 24 hours of exposure, while their effect in body deformations of newborns was

337   even synergistic. Ondracek et al. (2012) exposed for 10 days Japanese quails for 10

338   days to a combination of a cyanobacterial biomass with MCs (61.62 µg/day MCs), the

339   organophosphate OP paraoxon (2 doses of 250 µg/Kg paraoxon in that period), and an

340   anticoagulant (2 doses of 500 mg/Kg bromadiolone). They observed that the

341   combination induced a more severe damages in the birds than the isolated compounds.

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342 This brings to light that the combination of cyanotoxins and pesticides usually drives to

343 a synergistic response, which is in agreement with our results at low concentrations

344 after 48 hours of exposure. In contrast, Asselman et al. (2013) observed non-interactive

345 and antagonistic effects for the combination of carbaryl with four species of

346 cyanobacteria (some of them CYN-producers) on *Daphnia pulex*. Moreover, when these

347 same authors studied the effects of the combination of four different insecticides

348 (including CPF) and *M. aeruginosa* on *Daphnia pulex* after 21 days of exposure, a

349 different response was found (Asselman et al., 2014). They observed an additive

350 responsehus, for CPF and the cyanobacterial strain they observed an additive response,

351 different to the antagonistic response that they found using carbaryl, which presents the

352 same mechanism of action (Asselman et al., 2013). For this reason, they postulated that

353 interactive effects could not be generalized for compounds targeting the same pathway

354 (Asselman et al., 2014).

355   Regarding the oxidative stress induction, the combination of CYN and CPF did

356 not induce a significant variation in ROS levels compared to the control group,

357 following the trend showed by CYN in Hinojosa et al. (2019a) and CPF described in the

358 present paper. Regarding the GSH levels, the combination of both compounds led to a

359 reduction of its levels. However, the decline was lower than the one caused by CPF

360 alone. This could be due to the fact that CYN alone did not induce any alteration on

361 GSH levels in SH-SY5Y cells (Hinojosa et al., 2019a). This decrease, as mentioned

362 before, could be the consequence of athe compensating mechanism for the oxidative

363 stress, explaining the non-altered ROS levels shown by the mixture. Up to date, no more

364 data concerning the oxidative stress induction of the combination of CYN and CPF *in*

365 *vitro* is available. However, the oxidative stress induction of both toxicants has been

366 assayed combined with other chemicals. The scientific literature is very scarce

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367 concerning to CYN. Our research group demonstrated that the combination of CYN

368 with MC-LR did not induce an alteration of ROS levels but a slight reduction of GSH

369 levels in SH-SY5Y cells (Hinojosa et al., 2019). This is in agreement with the results

370 presented in this paper, being worthy to point out that CYN ameliorates GSH levels in

371 the mixture compared to CPF alone. Regarding CPF, its combination with other

372 chemicals using SH-SY5Y cells as *in vitro* model has resulted in different outcomes.

373 Thus, Raszewski et al. (2015) found that the combination of CPF-cypermethrin, a

374 synthetic pyrethroid, induced a synergistic response in the MTT assay, whereas a

375 mixture of CPF and Cd presented an antagonistic response, not showing a deterioration

376 of the oxidative stress status previously induced by CPF alone (Xu et al., 2017). These

377 results support the theory that CYN could be the limiting factor of the combination.

378 Concerning the AChE status, the combination of CYN and CPF showed a

379 similar pattern than CPF alone, thus is, an intense inhibition of the AChE activity. This

380 same fact was described by Xu et al. (2017) in SH-SY5Y cells, who found that

381 cadmium had no effect on the inhibitory AChE activity in combination with CPF. This

382 result reveals, for the first time, that in a CYN-CPF mixture, CPF is the one that mainly

383 determines the inhibition of the AChE enzyme.

384 Finally, regarding to the morphological findings, Hinojosa et al. (2019a) showed

385 that CYN induced clear morphological alterations leading to cell death (apoptotic

386 bodies, heterochromatin condensation, nuclei with an irregular shape, segregated

387 nucleolus, and numerous mitochondria in the cytoplasm). These observations were very

388 similar to those observed for CPF. Thus, the induction of mitochondrial dysfunction

389 observed after CPF exposure, could be explained not only by its intrinsic pro-oxidant

390 capacity, but also by altering mitochondrial architecture and dynamics (de Oliveira et

391 al., 2016). The combination of CYN and CPF showed a more intense cell death by

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392 apoptosis than the individual exposure to CYN and CPF alone. This result is in

393 agreement with those reported by Raszewski et al. (2015) and Xu et al. (2017) in SH-

394 SY5Y cells exposed to a combination of CPF and cypermethrin, and CPF and Cd,

395 respectively. In the last case, the observations at mitochondrial levels were very similar

396 to those described in the present paper, with swelling and fragmentation.

397

5.   Conclusions

399 Our findings indicate that the combination of CYN and CPF induces GSH

400 depletion, AChE activity inhibition, and cell death by apoptosis in the human

401 neuroblastoma SH-SY5Y cell line. In comparison to CYN and CPF alone, an

402 intensification of these effects was observed in this cell line after exposure to the

403 mixture. However, these observations were less severe than expected, which was

404 corroborated by the isobologram method. Thus, a mainly antagonistic response was

405 established between both compounds. Nevertheless, a synergistic effect between both

406 substances was observed at low concentrations after 48 hours, which requires special

407 attention in order to not underestimate the exposure concentrations. Hence, from a

408 toxicological point of view, it is important to consider the exposure pattern (single or

409 mixtures) as results can differ. Further research is required to contribute to the risk

410 assessment of CYN and other contaminants considering more realistic exposure

411 scenarios.

412

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1   *In vitro* assessment of the combination of Cylindrospermopsin and the

2   organophosphate chlorpyrifos on the human neuroblastoma SH-SY5Y cell line

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Abstract

29

30   Cylindrospermopsin (CYN) is a cyanotoxin which occurrence is increasing due

31   to climate change. This toxin is able to exert damage in the organism at several levels,

32   among them, in the nervous system. Moreover, it is important to take into account that

33   this toxin is not usually present isolated in nature, but in combination with some other

34   pollutants, being the case of the pesticide chlorpyrifos (CPF). Thus, the aim of the

35   present work was to assess the effects of the interaction of CYN in combination with

36   CPF in the human neuroblastoma cell line SH-SY5Y by evaluating cytotoxicity and

37   mechanistic endpoints. The mixtures 0.25+21, 0.5+42, 1+84 µg/mL of CYN+CPF

38   based on cytotoxicity results, were evaluated, and the isobologram method detected an

39   antagonistic effect after 24 and 48 hours of exposure. Moreover, although no alterations

40   of reactive oxygen species were detected, a significant decrease of glutathione levels

41   was observed after exposure to both, CPF alone and the combination, at all the

42   concentrations and times of exposure assayed. In addition, CYN + CPF caused a

43   marked decrease in the acetylcholinesterase activity, providing similar values to CPF

44   alone. However, these effects were less severe than expected. All these findings,

45   together with the morphological study results obtained, point out that it is important to

46   take into account the interaction of CYN with other pollutants. Further research is

47   required to contribute to the risk assessment of CYN and other contaminants

48   considering more realistic exposure scenarios.

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51   **Keywords**: Cylindrospermopsin, chlorpyrifos, SH-SY5Y cells, chemicals combination,

52   pollutants

2

1. Introduction

Cyanobacterial blooms are a global environmental concern due to their increased occurrence in terrestrial, marine and freshwater ecosystems, caused by climate change and human influence. Among their detrimental effects, they produce unpleasant organoleptic properties where they proliferate (Manning and Nobles, 2017). However, the main hazard is their capacity to produce cyanotoxins, toxic secondary metabolites that threaten water safety and aquatic life (Brooks et al., 2017; Buratti et al., 2017). There are different exposure routes to them, standing out the oral one as one of the most common and dangerous. Nonetheless, other relevant routes such as dermal contact or inhalation are worth to be mentioned as well (Buratti et al., 2017).

Cylindrospermopsin (CYN) is one of the most common aquatic cyanotoxins as a consequence of the increasing occurrence of *Cylindrospermopsis raciborskii* blooms, the most relevant producer species (Antunes et al., 2015). However, other cyanobacterial species such as *Chrysosporum ovalisporum, Aphanizomenon flos-aquae, Anabaena bergii,* or *Raphidiopsis curvata* are able to produce this toxin as well (Manning and Nobles, 2017). Cylindrospermopsin consists of an alkaloid with a tricyclic guanidine linked to a hydroxymethyl uracil group (Ohtani et al., 1992). The main episode of human intoxication by CYN occurred in the outbreak of 1979 in Palm Island (Australia) when 146 people were hospitalized in a local clinic with malaise, vomits, anorexia and hepatomegaly after drinking from a water supply containing CYN-producing *C. raciborskii* (Bourke et al., 1983; Griffiths and Saker, 2003). However, despite intoxication by drinking contaminated water, it is also feasible to be exposed by ingestion of contaminated food. In fact, the bioaccumulation of these cyanotoxins by plants irrigated with contaminated water (Testai et al., 2016) or in exposed aquatic organisms has been demonstrated, enhancing the risk of human intoxication by the food

3

78   chain (Cordeiro-Araújo et al., 2017; Gutiérrez-Praena et al., 2013; Machado et al.,

79   2017).

80   Although its target organ is the liver, CYN has widely demonstrated its capacity

81   to damage other organs, including the nervous system (Guzmán-Guillén et al., 2015;

82   Falconer, 1999; Hawkins et al., 1985; Humpage et al., 2005; Terao et al., 1994). Its

83   main mechanism of action is the irreversible inhibition of protein and glutathione

84   (GSH) synthesis (Froscio et al., 2003). Moreover, CYN has demonstrated its ability to

85   increase reactive oxygen species (ROS) production, linked to apoptosis and DNA

86   damage (Guzmán-Guillén et al., 2013; Puerto et al., 2011), and progenotoxic effects in

87   several cell lines (Puerto et al., 2018; Žegura et al., 2011). In addition, several studies

88   have shown the capacity of CYN to cause neuronal damage *in vitro* (Hinojosa et al.,

89   2019a; Takser et al., 2016) and *in vivo* (da Silva et al., 2018; Guzmán-Guillén et al.,

90   2015), by inducing changes in the acetylcholinesterase activity (AChE) and oxidative

91   stress (Hinojosa et al., 2019b).

92   Furthermore, the pollution of water by pesticides and their metabolites has been

93   of great concern due to the increasing number of pesticides used in agricultural practices

94   and detected in water, as well to their persistence, mobility, and toxicity (Carvalho,

95   2012). Among all types of pesticides, organophosphates (OP) are about 38% of the

96   global pesticides used, due to their high effectiveness against target pests and relatively

97   low toxicity to non-target organisms (Koly and Khan, 2018). One of the most common

98   OP used in agriculture and residential pest is chlorpyrifos (CPF) [O,O-diethyl 0-(3, 5, 6-

99   trichloro-2-pyridinol) phosphorothionate] (Mehta et al., 2009). This OP has been

100   detected in many samples from all over the world (Koly and Khan, 2018).

101   The cases of human intoxication with CPF are numerous, including symptoms of

102   headache, dizziness, nausea, sweating, salivation, muscle twitching, unconsciousness,

4

convulsion, and death (Eaton et al., 2008). The main mechanism of action for CPF is the

inhibition of the acetylcholinesterase activity, which leads to accumulation of

acetylcholine in the synaptic space, causing an excessive stimulation of postsynaptic

neuronal receptors and consequent signs of toxicity (Al-Badrany and Mohammad, 2007;

Mehta et al., 2009; Zheng et al., 2000). Moreover, CPF induces changes on

macromolecule synthesis (DNA, RNA, proteins), on neurotransmitter receptors, and in

signal transduction pathways, neuronal differentiation, and neurochemical effects.

Furthermore, it is also capable of producing oxidative stress by lipid peroxidation and

increasing ROS (Uchendu et al., 2012).

       Recently, the European Food Safety Authority has established the need of

studying the toxicity of the mixtures of cyanotoxins and other chemicals, as some OP

pesticides were reported to potentiate the anatoxin-induced toxicity, for instance (Cook

et al., 1988, Testai et al., 2016). In this sense, due to the possible coexistence of CYN

and CPF in water or their bioaccumulation in aquatic organisms or crops, the aim of the

present work was to study, for the first time, the potential interaction and effects of the

combination of the cyanotoxin CYN and the pesticide CPF in the human neuroblastoma

cell line SH-SY5Y, including cytotoxicity, oxidative stress, AChE inhibition, and

morphological changes.

## 2.	Materials & Methods

2.1. Supplies and chemicals

       Cylindrospermopsin (purity > 95% by HPLC) was purchased from Enzo Life

Sciences. Nutrient Mixture F-12 Ham and CPF were purchased from Sigma-Aldrich

126 (Madrid, Spain). Minimum essential medium (MEM), fetal bovine serum (FBS) and

127 cell culture reagents were obtained from Gibco (Biomol, Sevilla, Spain).

128   The Bradford reagent was purchased from Sigma-Aldrich (Madrid, Spain). The

129 supplier of MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

130 sulphophenyl)-2H-tetrazolium salt) Cell Titer 96® AQueous One Solution Cell

131 Proliferation Assay was Promega (Biotech Iberica, Madrid, Spain).

132 *2.2. Model system*

133   SH-SY5Y cells, derived from a human neuroblastoma, were obtained from the

134 American Type Culture Collection (CRL-2266). These cells were maintained in a MEM

135 and F-12 (1:1) medium supplemented with 10% FBS, 1% L-glutamine 200 mM, 1%

136 sodium pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin

137 solution, in an atmosphere containing 5% CO_2 at 95% relative humidity at 37ºC (CO_2

138 incubator, NuAire®, Spain). Cells were grown 80% confluent in 75-cm^2 plastic flasks

139 and harvested twice a week with 0.25% trypsin-EDTA (1X). The quantification of the

140 cells was performed in a Neubauer chamber. SH-SY5Y cells were plated at density of

141 $1 \cdot 10^5$ cells/mL to perform all the experiments.

142 *2.3. Toxin test solutions*

143   Stock solution of 1 mg/mL CYN was prepared in sterilized milliQ water, and

144 maintained at -20˚C until its use. Stock solution of 50 mg/mL CPF was prepared in

145 absolute ethanol and maintained at -20˚C until its use.

146 *2.4. Cytotoxicity assays*

147   SH-SY5Y cells were seeded for basal toxicity tests in 96-well tissue-culture

148 plates and, after being incubated at 37˚C for 24 hours, the exposure took place. From

149 the CYN-stock solution, serial dilutions in medium without serum were prepared (0-1

150 μg/mL CYN) based on previous studies (Hinojosa et al., 2019a). From the stock

151 solution of CPF, serial dilutions in medium without serum were prepared (0-200 μg/mL

152 CPF). This concentration was selected based on previous studies (Amani et al., 2016;

153 Fu et al., 2019). Non-treated cells were used as negative control. A solvent control was

154 also evaluated to ensure its innocuous role (data not shown). After replacing the

155 medium, the addition of the exposure solutions to the plates and incubation for 24 and

156 48 hours at 37°C took place. The endpoints assayed were protein content (PC) and

157 tetrazolium salt reduction (MTS). All the assays were performed by triplicate. All the

158 endpoints assayed are well described in Hinojosa et al. (2019a).

159 *2.5. Assessment of the effect of CYN-CPF combination by the isobolograms method*

160   Concentrations used to evaluate the toxic potential of the combination CYN-

161 CPF were chosen based on the cytotoxicity results of CYN and CPF previously

162 obtained (Hinojosa et al., 2019a). The highest exposure concentrations for the

163 combination studies were those obtained as the mean effective concentration (EC_{50}) of

164 the most sensitive endpoint, in this case, the MTS assay at 24 hours, along with the

165 fractions $EC_{50}/2$ and $EC_{50}/4$. Thus, SH-SY5Y cells were exposed for 24 and 48 hours to

166 binary pure pollutant combinations: EC_{50} CYN + EC_{50} CPF, $EC_{50}/2$ CYN + $EC_{50}/2$ CPF

167 and $EC_{50}/4$ CYN + $EC_{50}/4$ CPF.

168   The isobologram method was used according to Tatay et al. (2014) to determine

169 the type of interaction occurring when CYN and CPF are combined. This method

170 consists of plotting the concentration-effects curves for each compound and their

171 combinations in multiple diluted concentrations using the median-effect equation. The

172 combination index (CI) quantifies the synergism (CI < 1), the additivity (CI = 1), or the

173 antagonism (CI > 1) of the combinations. The CalcuSyn software calculates these CI

174 values automatically (Biosoft, Cambridge, UK).

175 *2.6. Oxidative stress assays*

176 *2.6.1. Reactive Oxygen Species (ROS) generation*

177 The ROS production was assessed in 96-well plates using the

178 dichlorofluorescein (DCF) assay, previously optimized by Medrano-Padial et al. (2019).

179 Fluorescence was measured at 535 nm (emission) and 485 nm (excitation) after

180 incubation for 4, 8, 12 and 24 hours.

181 *2.6.2. Glutathione (GSH) content*

182 The GSH content was measured according to Maisanaba et al. (2018). Cells

183 were exposed and incubated for 4, 8, 12 or 24 hours. Buthionine sulfoximine (BSO) 1μM

184 was used as positive control. Fluorescence was measured 460 nm (emission) and 380

185 nm (excitation).

186 *2.7. Acetylcholinesterase (AChE) activity determination*

187 Acetylcholinesterase activity was measured following the method of Ellman et

188 al. (1961) with modifications (Santillo et al., 2015). Viable SH-SY5Y cells were

189 exposed to the toxins and incubated at 37ºC for 24 hours. A solution of parathion 50 nM

190 was used as a positive control. Then, the medium was discarded and replaced by 200 μL

191 of a reaction mixture containing 0.5 mM 5,5-dithio-bis-(2-nitrobenzoic acid) and 100

192 μM acetyltiocholine per well. The resulting product of the reaction, 5-thio-2-

193 nitrobenzoate, was measured at 410 nm every 90 seconds up to 1 hour.

194 *2.8. Morphology*

195 The concentrations used for the morphological study were the ones obtained in

196 the cytotoxicity assays, being the EC_{50} values those obtained after 24 hours of exposure.

197 These values were chosen as the highest concentrations of exposure along with the

198 fractions $EC_{50}/2$ and $EC_{50}/4$. SH-SY5Y cells were exposed for 24 hours. The procedures

199   for the contrast, light and electron microscopies were described by Hinojosa et al.

200   (2019a).

201   *2.10. Calculations and statistical analysis*

202   Data for the cytotoxicity assays, oxidative stress biomarkers and AChE were

203   presented as mean ± standard deviation (SD) to control. Analysis of variance (ANOVA)

204   followed by Dunnett's multiple comparison was carried out to perform the statistical

205   analysis, using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

206   Differences were considered significant from $P<0.05$. EC_{50} values were the result of the

207   linear regression in the concentration-response curves.

208   **3.   Results**

209   *3.1. Cytotoxicity assays*

210   A concentration-dependent decrease in the viability was observed in the SH-

211   SY5Y cells after being exposed to 0-200 µg/mL CPF for 24 and 48 hours (Fig. 1). The

212   EC_{50} values obtained were 83.98 ± 2.74 µg/mL and 85.31 ± 7.67 µg/mL after 24 hours

213   of exposure for the MTS assay and the PC assay, respectively. In the case of the cells

214   exposed to CPF for 48 hours, the EC_{50} values were 69.78 ± 6.02 µg/mL and >200

215   µg/mL for the MTS and PC assays, respectively. Thus, the MTS assay demonstrated to

216   be the most sensitive biomarker.

217   *3.2. Assessment of the effect of CYN-CPF combination by the isobolograms method*

218   Using the EC_{50} value previously indicated in Hinojosa et al. (2019a), the

219   exposure concentrations of CYN were 0.25, 0.5 and 1 µg/mL, and 21, 42 and 84 µg/mL

220   of CPF, being both combined in a proportion of 1:84 for 24 or 48 hours (Fig. 2). The

221   experiments performed with the combination presented a CI>1 in the case of the cells

222   exposed for 24 hours, confirming an antagonistic response (Fig. 3A). However, in those

9

223   cells exposed for 48 hours, the response obtained was mainly synergic at low

224   concentrations (CI<1) and antagonistic at higher ones (CI>1) (Fig. 3B).

225   *3.3. Oxidative stress assays*

226   The exposure to 21, 42 or 84 µg/mL CPF led to no significant changes in the

227   ROS levels compared to the control group in SH-SY5Y cells after any of the times of

228   exposure (4, 8, 12 or 24 hours) (Fig. 4A). However, it caused a significant decrease of

229   the GSH levels after all the times of exposure to the three concentrations assayed (Fig.

230   4B).

231   Similarly, the combination of the cyanotoxin and the OP (1:84) caused a

232   reduction of the GSH levels in all the concentrations and times of exposure assayed

233   (Fig. 4D), despite the ROS levels were not altered compared to the control group (Fig.

234   4C).

235   *3.4. Acetylcholinesterase activity*

236   All the concentrations of CPF (21, 42 and 84 µg/mL) reduced the AChE activity

237   in SH-SY5Y cells. Concerning the combination, all the concentrations assayed induced

238   a reduction of the AChE activity (Fig. 5).

239   *3.5. Morphology study*

240   Unexposed SH-SY5Y cells observed under phase-contrast microscopy showed a

241   star-shape morphology with neurites contacting adjacent cells. The presence of mitotic

242   processes evidenced the active proliferation of these cells (Fig. 6A). The light

243   microscope revealed clear euchromatic nuclei with obvious nucleoli (Fig. 6B). Under

244   electronic microscope, cells showed a big number of mitochondria in the cytoplasm,

245   which also presented vacuole with a dense matrix (Fig. 6C).

246   *3.5.1. Microscope observations of cells exposed to CPF*

247   Cells exposed to 42.5 and 84 µg/mL CPF presented, under phase-contrast and

248   light microcopies, cell death signs. Cytoplasmic projections diminished with a low

249   proliferation rate. At the lowest concentration, a vacuolization of the cytoplasm was

250   observed (Fig. 6D-E). Ultrastructurally, mitochondrial organelles increased their size

251   due to the entrance of water. In addition, an alteration of the pattern of the

252   mitochondrial crests was observed. These cellular processes drove to the presence of

253   heterophagosomes and condensation of the chromatin, a clear sign of apoptosis (Fig.

254   6F).

255   *3.5.2. Microscope observations of cells exposed to the combination CYN+CPF*

256   Concerning the combination, intense vacuolization of the cytoplasm, cellular

257   cycle detention, loss of cytoplasmic projections, and cell death were observed at the

258   three concentrations assayed (Fig. 6G-H). Under transmission electronic microscopy,

259   small lipid droplets associated with mitochondrial organelles were observed. Moreover,

260   at the highest dose assayed (1 µg/mL CYN + 84 µg/mL CPF), rough endoplasmic

261   reticulum (RER) cisternae appeared dilated with dense content, and an intense presence

262   of heterophagosomes was observed (Fig. 6I).

263

264   **4.   Discussion**

265   The interactive effects of chemicals and natural stressors have been reviewed

266   (Holmstrup et al., 2010; Laskowski et al., 2010). Synergistic interactions were reported

267   in more than 50% of the available studies of interactions between chemicals with abiotic

268   stressors. The coexistence of multiple cyanotoxins or with some other active

269   compounds has been described (Al-Sammak et al., 2014; Chen et al., 2017; Martínez-

270   Ruiz and Martínez-Jerónimo, 2016; Pathmalal, 2019; Tatters et al., 2017). However,

271 although pesticides are candidates to appear together with cyanotoxins, literature

272 regarding this subject is almost non-existent (Asselman et al., 2014; Daam et al., 2011;

273 Moe et al., 2012). In this sense, EFSA has highlighted the importance of addressing the

274 concomitant exposure of other chemicals, such as pesticides, along with cyanotoxins in

275 order to have information on mixture toxicity and on the environmental factors affecting

276 toxicity of a bloom (Testai et al., 2016).

277   The present work focused on the potential effects induced by the combination of

278 the cyanotoxin CYN and the organophosphate CPF in the neuronal SH-SY5Y cell line.

279 To address this research, a cytotoxicity study of CYN and CPF in the cell model was

280 required to establish the EC_{50} values of the test items to be applied in the combination

281 study. In the case of CYN, the EC_{50} value of 1 µg/mL CYN was taken from a previous

282 work, where SH-SY5Y cells were exposed to 0-10 µg/mL CYN for 24 and 48 hours

283 (Hinojosa et al., 2019a). Regarding CPF our study confirms its damaging properties in

284 neuronal cells, showing a time- and concentration-dependent cytotoxicity. In agreement

285 with our results, Park et al. (2013) demonstrated that CPF induced cytotoxic effects in a

286 concentration-dependent manner in SH-SY5Y cells exposed to 0-70 µM CPF,

287 establishing an EC_{50} value of ~32 µg/mL CPF after 24 hours of exposure. Similarly,

288 Raszewski et al. (2015) also found a time- and concentration-dependent cytotoxic

289 behavior of CPF in SH-SY5Y cells exposed during 24, 48, and 72 hours to 0-175

290 µg/mL CPF, obtaining an EC_{50} value for 24 hours of ~100 µg/mL CPF.

291   The potential mechanisms by which CPF induces its neurotoxic effects are

292 oxidative stress and acetylcholinesterase inhibition, among others (Park et al., 2013;

293 2015). In our study, CPF did not show any alteration of ROS levels at any of the

294 concentrations assayed. However, GSH levels presented a concentration-dependent

295 decrease, which could indicate that GSH is scavenging ROS. In this sense, Park et al.

296   (2015) and Xu et al. (2017) found an increment in ROS levels when SH-SY5Y cells

297   were exposed to CPF. However, they did not evaluate the GSH levels, which could give

298   an idea about the antioxidant activity of the neuroblastoma cell line. On the other hand,

299   Giordano et al. (2007) evaluated GSH levels of SH-SY5Y cells exposed to CPF. The

300   authors did not find alterations in the tripeptide levels, although they observed and

301   increment in oxidized GSH levels, which indicated that an antioxidant response was

302   taking place, suggesting that cytotoxicity may be mediated by oxidative stress. In

303   addition, Park et al. (2013, 2015) suggested that CPF-oxidative stress induction was

304   related to AChE inhibition, together with its usual mechanism of action. In agreement

305   with this, CPF induced a clear inhibition of the AChE activity at all the concentrations

306   assayed in this case. Other authors have also pointed out that a depletion of GSH levels

307   is linked to mitochondrial dysfunction, which could lead to apoptotic processes (de

308   Oliveira et al., 2016; Park et al., 2013). This is in concordance with our morphological

309   findings, where the highest concentrations of CPF induced mitochondrial alterations,

310   leading to cell death by apoptosis. This process is related to the activation of caspase-9

311   and -3 and an increment of cytosolic cytochrome c, as Park et al. (2015) reported for

312   SH-SY5Y cells exposed to CPF. Considering this, Raszewski et al. (2015) proposed that

313   CPF induces neurotoxicity through apoptotic mechanisms.

314   Once the toxic effects of CYN (Hinojosa et al., 2019a) and CPF alone on the

315   neuronal SH-SY5Y cell line were elucidated, the behavior of their combination on the

316   same cell line was evaluated. Our results showed that the combination was more

317   cytotoxic than both toxicants alone after 24 and 48 hours. However, in order to establish

318   the type of interaction between both compounds, the isobologram method was used.

319   Thus, after 24 hours of exposure, an antagonistic response was observed. This pattern

320   changed after 48 hours, showing a synergistic response at low concentrations, and

13

321 turning to an antagonistic response at higher concentrations. A similar pattern was

322 found when the combined toxicity of the cyanotoxin Microcystin (MC)-LR and Cu

323 exposure was investigated in the aquatic plant *Vallisneria natans*, producing synergistic

324 effects when combined at low concentrations (Wang et al., 2017). Considering this, it is

325 worth to mention that CYN usually appears at low concentrations in nature (de la Cruz

326 et al., 2013), making a synergistic toxic response very likely to appear.

327   To our knowledge, no studies concerning the effects of the combination of

328 cyanotoxins and pesticides using neuronal cell lines have been carried out. However, *in*

329 *vivo,* the first combination of cyanobacteria and pesticides was performed by Cerbin et

330 al. (2010), using a strain of *Microcystis aeruginosa* and the carbamate carbaryl. In this

331 study, the combination demonstrated a mostly additive interaction in *Daphnia pulicaria*

332 after 24 hours of exposure, while their effect in body deformations of newborns was

333 even synergistic. Ondracek et al. (2012) exposed Japanese quails for 10 days to a

334 combination of a cyanobacterial biomass with MCs (61.62 μg/day MCs), the OP

335 paraoxon (2 doses of 250 μg/Kg paraoxon in that period), and an anticoagulant (2 doses

336 of 500 mg/Kg bromadiolone). They observed that the combination induced more severe

337 damages in the birds than the isolated compounds. This brings to light that the

338 combination of cyanotoxins and pesticides usually drives to a synergistic response,

339 which is in agreement with our results at low concentrations after 48 hours of exposure.

340 In contrast, Asselman et al. (2013) observed non-interactive and antagonistic effects for

341 the combination of carbaryl with four species of cyanobacteria (some of them CYN-

342 producers) on *Daphnia pulex*. Moreover, when these same authors studied the effects of

343 the combination of four different insecticides (including CPF) and *M. aeruginosa* on

344 *Daphnia pulex* after 21 days of exposure, a different response was found (Asselman et

345 al., 2014). They observed an additive response for CPF and the cyanobacterial strain,

14

346 different to the antagonistic response that they found using carbaryl, which presents the

347 same mechanism of action (Asselman et al., 2013). For this reason, they postulated that

348 interactive effects could not be generalized for compounds targeting the same pathway

349 (Asselman et al., 2014).

350   Regarding the oxidative stress induction, the combination of CYN and CPF did

351 not induce a significant variation in ROS levels compared to the control group,

352 following the trend showed by CYN in Hinojosa et al. (2019a) and CPF described in the

353 present paper. Regarding the GSH levels, the combination of both compounds led to a

354 reduction of its levels. However, the decline was lower than the one caused by CPF

355 alone. This could be due to the fact that CYN alone did not induce any alteration on

356 GSH levels in SH-SY5Y cells (Hinojosa et al., 2019a). This decrease, as mentioned

357 before, could be the consequence of a compensating mechanism for the oxidative stress,

358 explaining the non-altered ROS levels shown by the mixture. Up to date, no more data

359 concerning the oxidative stress induction of the combination of CYN and CPF *in vitro*

360 is available. However, the oxidative stress induction of both toxicants has been assayed

361 combined with other chemicals. The scientific literature is very scarce concerning to

362 CYN. Our research group demonstrated that the combination of CYN with MC-LR did

363 not induce an alteration of ROS levels but a slight reduction of GSH levels in SH-SY5Y

364 cells (Hinojosa et al., 2019). This is in agreement with the results presented in this

365 paper, being worthy to point out that CYN ameliorates GSH levels in the mixture

366 compared to CPF alone. Regarding CPF, its combination with other chemicals using

367 SH-SY5Y cells as *in vitro* model has resulted in different outcomes. Thus, Raszewski et

368 al. (2015) found that the combination of CPF-cypermethrin, a synthetic pyrethroid,

369 induced a synergistic response in the MTT assay, whereas a mixture of CPF and Cd

370 presented an antagonistic response, not showing a deterioration of the oxidative stress

15

371 status previously induced by CPF alone (Xu et al., 2017). These results support the

372 theory that CYN could be the limiting factor of the combination.

373   Concerning the AChE status, the combination of CYN and CPF showed a

374 similar pattern than CPF alone, thus is, an intense inhibition of the AChE activity. This

375 same fact was described by Xu et al. (2017) in SH-SY5Y cells, who found that

376 cadmium had no effect on the inhibitory AChE activity in combination with CPF. This

377 result reveals, for the first time, that in a CYN-CPF mixture, CPF is the one that mainly

378 determines the inhibition of the AChE enzyme.

379   Finally, regarding the morphological findings, Hinojosa et al. (2019a) showed

380 that CYN induced clear morphological alterations leading to cell death (apoptotic

381 bodies, heterochromatin condensation, nuclei with an irregular shape, segregated

382 nucleolus, and numerous mitochondria in the cytoplasm). These observations were very

383 similar to those observed for CPF. Thus, the induction of mitochondrial dysfunction

384 observed after CPF exposure could be explained not only by its intrinsic pro-oxidant

385 capacity but also by altering mitochondrial architecture and dynamics (de Oliveira et al.,

386 2016). The combination of CYN and CPF showed a more intense cell death by

387 apoptosis than the individual exposure to CYN and CPF. This result is in agreement

388 with those reported by Raszewski et al. (2015) and Xu et al. (2017) in SH-SY5Y cells

389 exposed to a combination of CPF and cypermethrin, and CPF and Cd, respectively. In

390 the last case, the observations at mitochondrial levels were very similar to those

391 described in the present paper, with swelling and fragmentation.

392

393 **5. Conclusions**

394   Our findings indicate that the combination of CYN and CPF induces GSH

395   depletion, AChE activity inhibition, and cell death by apoptosis in the human

396   neuroblastoma SH-SY5Y cell line. In comparison to CYN and CPF alone, an

397   intensification of these effects was observed in this cell line after exposure to the

398   mixture. However, these observations were less severe than expected, which was

399   corroborated by the isobologram method. Thus, a mainly antagonistic response was

400   established between both compounds. Nevertheless, a synergistic effect between both

401   substances was observed at low concentrations after 48 hours, which requires special

402   attention in order to not underestimate the exposure concentrations. Hence, from a

403   toxicological point of view, it is important to consider the exposure pattern (single or

404   mixtures) as results can differ. Further research is required to contribute to the risk

405   assessment of CYN and other contaminants considering more realistic exposure

406   scenarios.

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A

CPF 24h

B

CPF 48h

Figure 2
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A

CYN + CPF 24h

% viability

	0	21	42	84	CPF
	0	0.25	0.5	1	CYN
	0	21 + 0.25	42 + 0.5	84 + 1	CPF + CYN
		EC₅₀/4	EC₅₀/2	EC₅₀	

Concentration (μg/mL)

B

CYN + CPF 48h

% viability

	0	21	42	84	CPF
	0	0.25	0.5	1	CYN
	0	21 + 0.25	42 + 0.5	84 + 1	CPF + CYN
		EC₅₀/4	EC₅₀/2	EC₅₀	

Concentration (μg/mL)

Figure 3
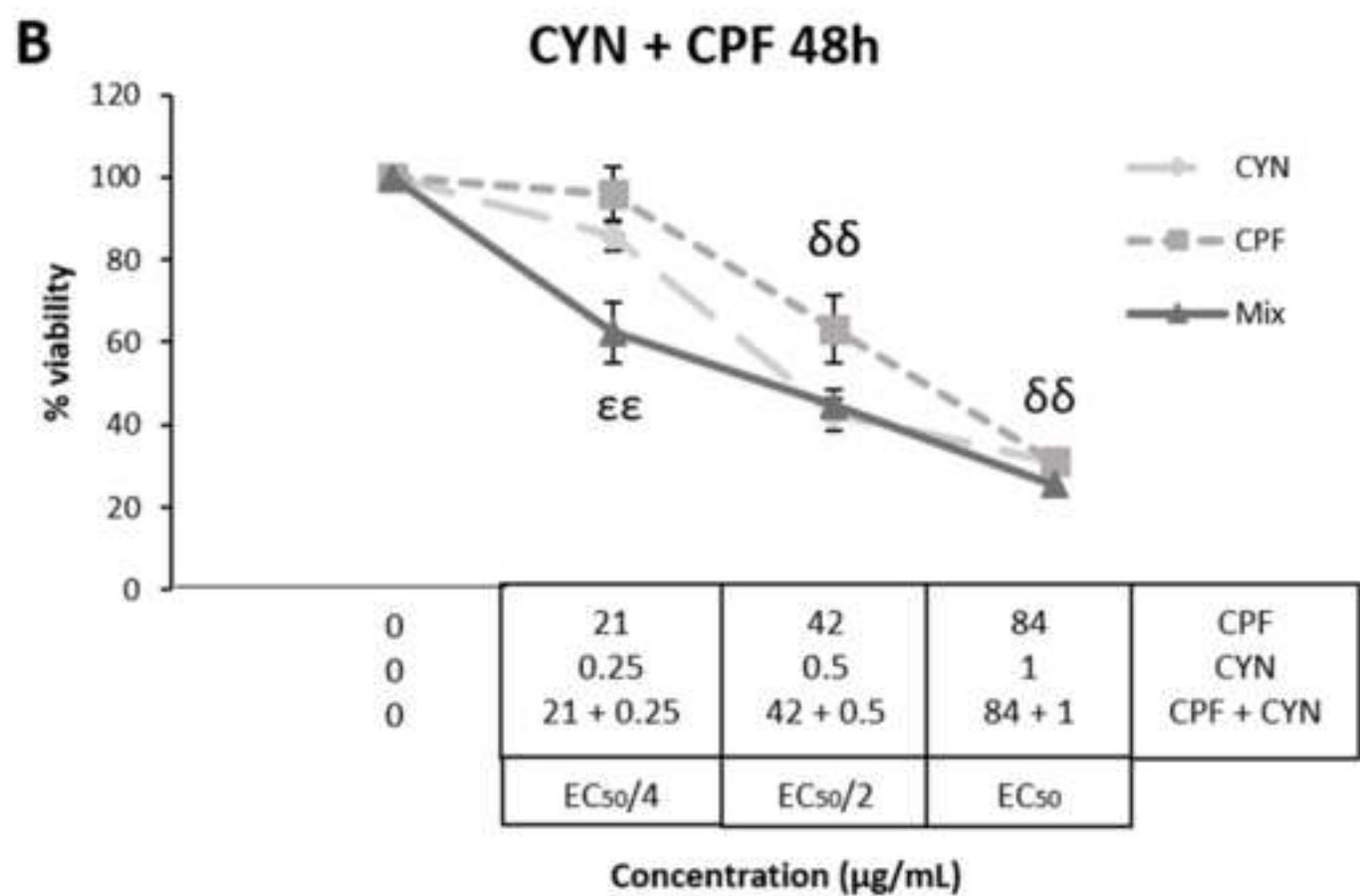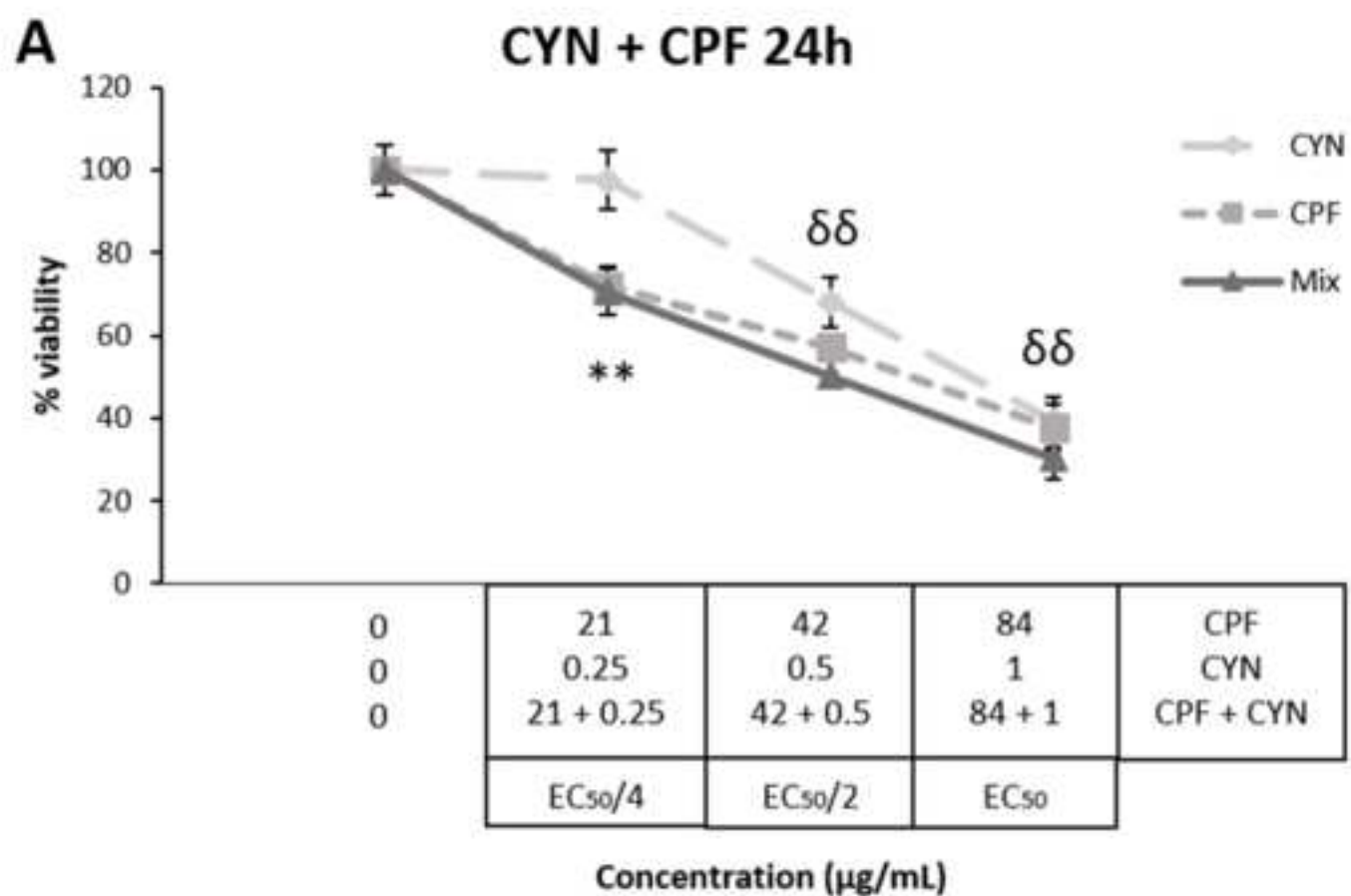
Figure 4

AChE

Figure 6
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Figure captions

Figure 1. Reduction of tetrazolium salt (MTS) and protein content (PC) on SH-SY5Y cells after 24 h (**A**) and 48 h (**B**) of exposure to 0-200 µg/mL CPF. All values are expressed as mean ± s.d. ** MTS significantly different from control group (p<0.01), §§ all parameters significantly different from control group (p<0.01).

Figure 2. Reduction of tetrazolium salt (MTS) on SH-SY5Y cells after 24 h (**A**) and 48 h (**B**) of exposure to different concentrations of CYN+CPF combinations at a ratio of EC_{50} CYN / EC_{50} CPF (1:84). All values are expressed as mean ± s.d. ** significantly different from control group (p<0.01) for CPF and the combination, εε significantly different from control group (p<0.01) for CYN and the combination, and δδ significantly different from control group (p<0.01) for CYN, CPF and the combination.

Figure 3. Combination index (CI)/fraction affected (Fa) curve in SH-SY5Y cells exposed to CYN-CPF combination after 24 h (**A**) and 48 h (**B**) of exposure. Each point represents the CI ± s.d. at a fractional effect. The dotted line (CI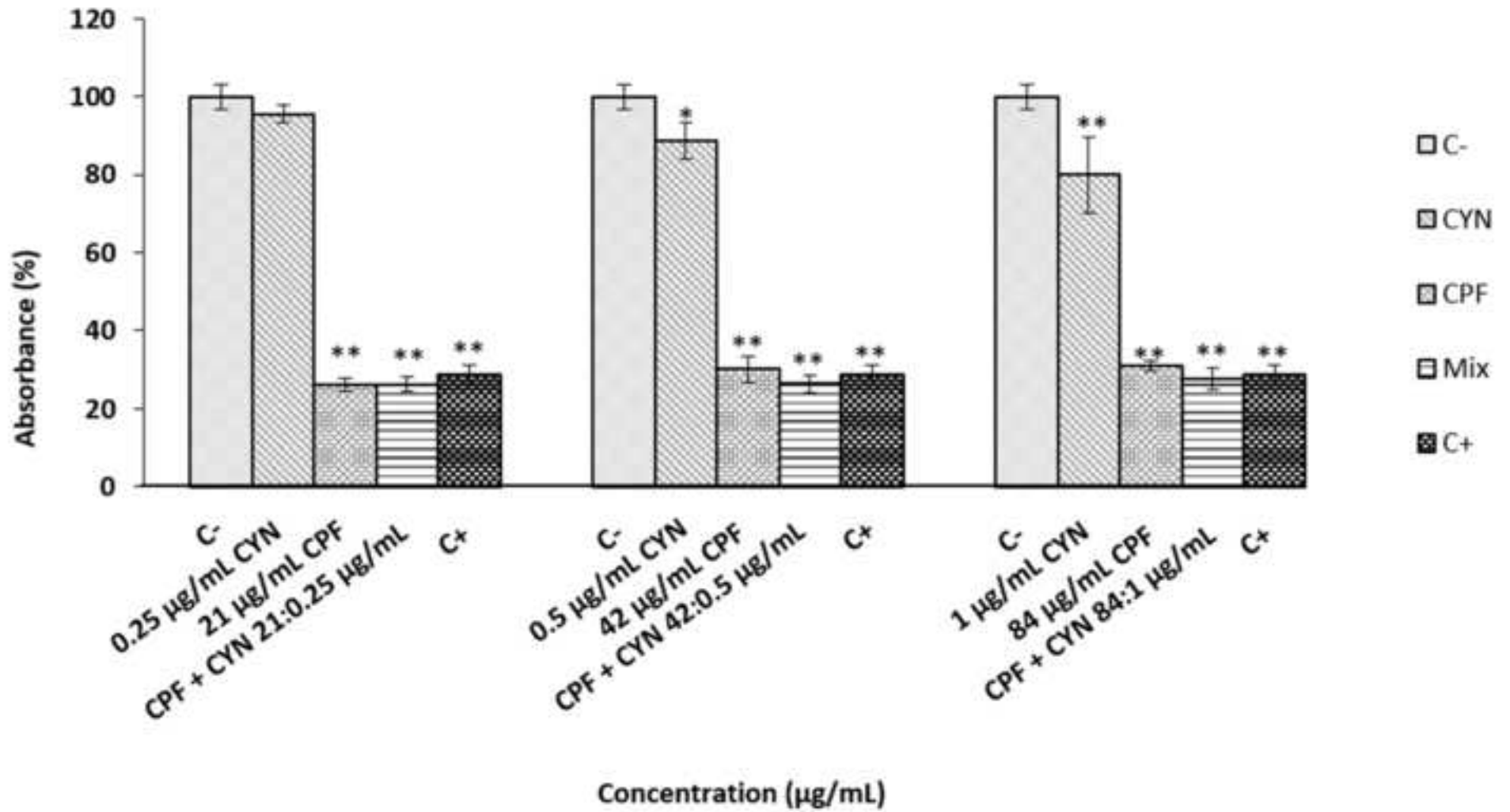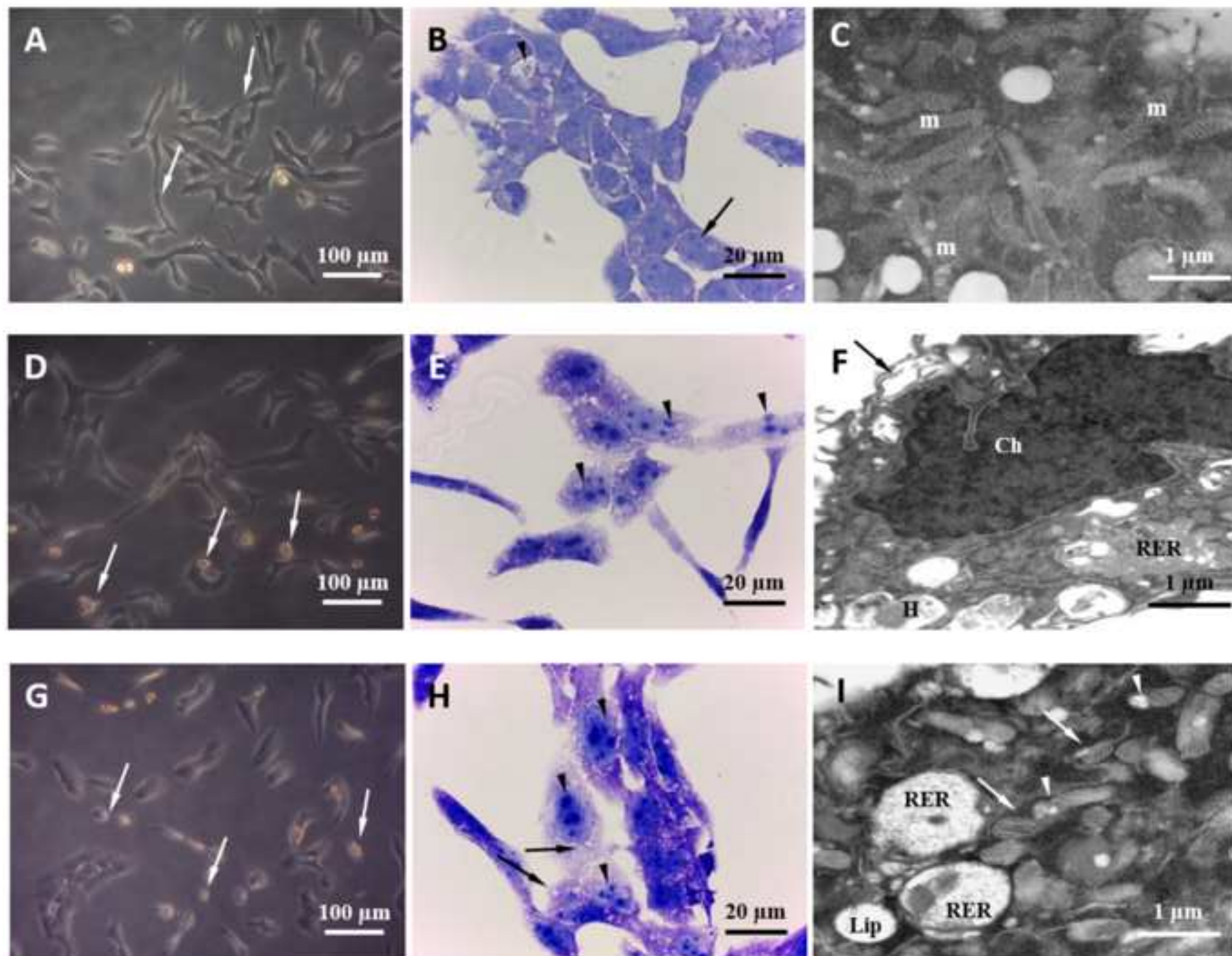 = 1) indicates additivity, the area under the dotted line points out a synergist effect, and the area above the dotted line signifies antagonism.

Figure 4. Reactive oxygen species (ROS) levels on SH-SY5Y cells after 4, 8, 12 and 24 hours of exposure to 0-84 µg/mL CPF (**A**) and a CYN-CPF combination at a ratio 1:84 of EC_{50} CYN / EC_{50} CPF (**C**). Reduced glutathione (GSH) levels on SH-SY5Y cells after 4, 8, 12 and 24 hours of exposure to 0-84 µg/mL CPF (**B**) and a CYN-CPF combination at a ratio 1:84 of EC_{50} CYN / EC_{50} CPF (**D**). All values are expressed as mean ± s.d. The significance levels observed are ** p<0.01 significantly different from control group.

Figure 5. *In vitro* effects of SH-SY5Y cells to their exposure to CYN (0-1 µg/mL), CPF (0-84 µg/mL) or CYN-CPF combination on the acetylcholinesterase activity (AChE) after 24 hours. All values are expressed as mean ± s.d. ** Indicates significant difference from control group value (p<0.01).

Figure 6. Morphology of control SH-SY5Y cells after 24 h of exposure to culture medium without serum. Contrast-phase microscopy of a SH-SY5Y cell culture in normal neuronal growth. Cells present cytoplasmic projections contacting with other cells (arrows) Bar=100µm (**A**). Semithin sections of cells culture were stained with toluidine blue. Cells in mitosis processes (arrowheads) with big nucleoli in the nucleus

(arrows). Bar=20μm (**B**). Transmission electronic microscopy of SH-SY5Y cells with numerous mitochondria (m). Bar=1 μm (**C**). Morphology of SH-SY5Y cells after 24 h of exposure to CPF. Phase-contrast microscopy of cells exposed to 42.5 μg/mL CPF presented rounded cells (arrows) with clear signs of cell death. Bar=100 μm (**D**). Semithin sections of cells exposed to 85 μg/mL CPF presented nuclei with condensed chromatin (arrowheads) as clear sign of cell death. Bar=20 μm (**E**). Electronic microscopy of cells exposed to 85 μg/mL CPF showed irregular nuclei with chromatin condensation (Ch), appearance of blisters in the cellular membrane (arrows), dilated RER with protein content (RER) and presence of heterophagosomes (H). Bar=1 μm (**F**). Phase-contrast microscopy of SH-SY5Y cells exposed to the combination of CYN and CPF showed cell death signs at all the concentrations assayed. Bar=100 μm (**G**). Semithin sections of cells culture exposed to 0.5 + 42 μg/mL CYN + CPF presented lipid droplets (arrows) and big nucleoli (arrowheads). Bar=20 μm (**H**). The TEM of cells exposed to the highest concentration of the combination showed altered dilated mitochondria (arrows) surrounded by lipidic droplets (arrowheads). The endoplasmic reticulum cisternae appeared dilated (RER) and lipidic vacuoles were formed (Lip). Bar=1 μm (**I**).

Credit Author Statement

- **María Gracia Hinojosa:** Conceptualization, Methodology, Formal Analysis, Investigation, Writing-Original Draft, Writing-Review & Editing, Visualization

- **Ana Isabel Prieto:** Conceptualization, Validation, Writing-Review & Editing, Supervision

- **Daniel Gutiérrez-Praena:** Conceptualization, Methodology, Validation, Formal Analysis, Writing-Original Draft, Writing-Review & Editing, Visualization, Supervision

- **Francisco Javier Moreno:** Validation, Investigation.

- **Ana María Cameán:** Conceptualization, Validation, Writing-Review & Editing, Supervision, Project administration, Funding adquisition.

- **Ángeles Jos:** Conceptualization, Validation, Writing-Review & Editing, Supervision, Project administration, Funding adquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

IV.   GENERAL DISCUSSION

1. Toxics isolated

Microcystin-LR is one of the most common MC-congeners worldwide and despite being considered a hepatotoxin, its potential to cause neurotoxicity has been also investigated in the last decades (Hu et al., 2016). The main mechanisms of action described for its neurotoxicity are PPs inhibition and Tau phosphorylation. This would lead to cytoskeletal disorganization and thus, to apoptosis. It also leads to oxidative stress and increase of intracellular calcium levels, which would also lead to apoptosis. In addition, the alteration of AChE activity and ACh, dopamine and GABA levels have also been reported, leading to central and peripheral nervous systems alterations (Hu et al., 2016). On the other hand, CYN is the second most common cyanotoxins worldwide (Yang et al., 2021). Although it has demonstrated to cause damage at different levels of the organism, its capacity to cause neurotoxicity has started to be investigated in the last decades. In this sense, CYN has been detected in brain of three different fish species, which indicated that this cyanotoxin is able to cross the blood-brain-barrier (Guzmán-Guillén et al., 2015; Da Silva et al., 2018; Rabelo et al., 2021). The main mechanisms of action implicated in its possible neurotoxicity would be cytoskeletal disorganization that, together with oxidative stress would lead to apoptosis, and alteration of the AChE activity. However, no studies have been performed using human cells up to now.

The nature of CPF is different compared to the other compounds studied in the present Doctoral Thesis, as is a human-made chemical used against plagues. Its main use is to affect the nervous system in the insects. Nonetheless, this OP has also demonstrated to exert toxicity in humans. In this regard, it is important to take into account the worldwide presence of this pesticide isolated first and in combination with some other possible environmental pollutants.

1.1. Cell viability

Concerning the cytotoxic effects of the toxics, MC-LR demonstrated to cause a concentration dependent decrease of both undifferentiated and differentiated SH-SY5Y cells in a concentration range from 0-100 µM after 24 and 48 hours of exposure. In the case of the undifferentiated cells, the EC_{50} values obtained were 36.21 ± 1.89 and 20.80 ± 2.08 µg/mL (37.91 and 21.78 µM) after 24 and 48 hours of exposure, respectively. However, in the differentiated SH-SY5Y, MC-LR led to EC_{50} values of 44.30 ± 0.91 and 37.01 ± 1.71 µg/mL (46.28 and 38.75 µM). These values would point out the differences in the effect in undifferentiated and differentiated cells, being the undifferentiated more sensitive to this cyanotoxin. This could be due to the need of the presence of OATP transporters for MC-LR to cross the cell membrane, as during the differentiation process, the levels of the different proteins and polypeptides may vary (Kovalevich and Langford, 2013). These results are in agreement to the ones obtained by Takser

et al. (2016) in murine microglia BV-2 cells and murine neuroblastoma N2a cells, who also reported a decrease in viability after exposure to 0-10 µM MC-LR for 24, 48 and 72 hours, being this decrease more prominent in the N2a cells. In this sense, the neuroblastoma cells demonstrated to be more sensitive to MC-LR than the microglia cells. Furthermore, in hipotalamic neuronal mouse GT1-7 cells, Ding et al. (2018) also reported a concentration-dependent decrease of the cell viability after 48 hours from 0.05 – 0.5 µM MC-LR.

Regarding, the cytotoxicity studies performed in undifferentiated SH-SY5Y cells exposed to pure CYN cause a concentration and time dependent-decrease of cell viability after 24 and 48 hours of exposure, leading to EC_{50} values of 0.87 ± 0.13 (2.09 µM) and 0.32 ± 0.08 µg/mL (0.77 µM), respectively. These values were much lower than the ones obtained after the exposure to MC-LR. Nonetheless, when the cells were exposed to a cyanobacterial extract of *C. ovalisporum* containing CYN (CYN+), the EC_{50} values obtained were 1.111 ± 0.325 (2.66 µM) and 0.691 ± 0.165 µg/mL (1.66 µM) after 24 and 48 hours, respectively. Thus, pure CYN demonstrated to exert higher toxicity than the extracts, suggesting the importance of taking into account not only the toxin itself but also some components that can be present when the real exposure to organisms occurs and that can interfere in the toxicity (EFSA, 2016). In order to assess the cytotoxic effects observed by CYN+ were due mainly to CYN and not some other cyanobacterial compounds, cells were also exposed to the same amount of extract of a cyanobacterial strain non containing CYN (CYN-), in this case a strain of *R. raciborskii*, which led to EC_{50} values of 5.658 ± 1.180 and 5.164 ± 1.620 µg/mL after 24 and 48 hours of exposure, respectively. In respect to the differentiated SH-SY5Y, as the lowest EC_{50} values were obtained after exposure to pure CYN, it was the one used for exposure to the differentiated cells, which led to a concentration-dependent decrease after 24 and 48 hours of exposure. In this experiment, the EC_{50} values obtained were 0.30 ± 0.05 (0.72 µM) and 0.53 ± 0.02 µg/mL (1.27 µM), respectively, also much lower than those obtained by MC-LR. This fact means that although the concentrations of CYN found in nature are lower than those of MC-LR, they have special toxicological relevance. Therefore, the differentiated cells were more sensitive to CYN after 24 hours of exposure, presenting the opposite response after 48 hours. This effect is the contrary to the one obtained by MC-LR, which demonstrated to exert higher toxicity in the undifferentiated cells compared to the differentiated ones. Concerning the studies performed in other cell lines, Takser et al. (2016) also demonstrated a concentration and time-dependent decrease of cell viability on the murine macrophage-like RAW246.7 cells, murine microglia BV-2 cells and murine neuroblastoma N2a cells after 24, 48 and 72 hours of exposure to 0.1 and 10 µM of pure CYN. These authors observed a reduction of cell viability to less than 20% in RAW246.7 and BV-2 cells after exposure to 10 µM for all the times of exposure assayed. However, 0.1 µM CYN had a higher impact of cell viability on the neuroblastoma N2a cells after all the concentrations of exposure.

With respect to the effects on cell viability of CPF, this pesticide led to concentration-dependent effects by providing EC_{50} values of 83.98 ± 2.74 and 85.31 ± 7.67 µg/mL (239.54 and 243.33 µM) after 24 and 48 hours of exposure, respectively in undifferentiated SH-SY5Y cell line, which is less cytotoxic than the response obtained by MC-LR and CYN. Nonetheless, in the differentiated cells, the values obtained were 33.51 ± 0.41 and 130.91 ± 1.75 µg/mL (95.58 and 373.4 µM), providing more cytotoxicity after 24 hours than MC-LR, but not after 48. All these values were obtained with the MTS assay, which demonstrated to be the most sensitive biomarker in all the cases. These data suggest that in the undifferentiated cells, CPF already exerts its toxicity during the first 24 hours of exposure and the cells cannot recover after another 24 hours, while in the case of the differentiated cells, despite presenting cytotoxicity after 24 hours of exposure, the cells might be able to recover. Therefore, it is important to take into account that the assay performed is based on mitochondrial viability, suggesting that the main cellular structure affected seems to be the mitochondria. In this line, Park et al. (2013) demonstrated also a concentration-dependent decrease in undifferentiated SH-SY5Y cells exposed to 0-70 µM, obtaining an EC_{50} value close to 100 µM (35 µg/mL), which is in agreement to the results that we obtained after 24 hours of exposure in the differentiated cells, but not in the undifferentiated. Nonetheless, Raszweski et al. (2015) also found a concentration- and time-dependent cytotoxic response in undifferentiated SH-SY5Y cells after exposure for 24, 48 and 72 hours to 0-175 µg/mL (0-500 µM), obtaining EC_{50} values of 313, 182 and 51 µM (109.73, 63.81 and 17.88 µg/mL), respectively after the MTT assay. In this regard, the results obtained in our study provide an EC_{50} after 24 hours in between those two reports.

Summarizing, the cytotoxicity assays demonstrate that MTS was the most sensitive biomarker, being CYN the most cytotoxic compound followed by MC-LR and CPF in the undifferentiated cell line. However, in differentiated SH-SY5Y, CPF demonstrated to exert higher toxicity after 24 hours of exposure than MC-LR, although CYN was again the most toxic compound. Furthermore, the differentiated cells demonstrated to be more sensitive for CYN, while MC-LR and CPF caused higher cytotoxicity in the undifferentiated. These differences could be due to changes in proteins and enzymes, together with changes in the metabolism during the differentiation process (Schneider et al., 2011).

1.2. Oxidative stress

Regarding the mechanisms of action of MC-LR, free-radical damage has demonstrated to be one of the most important pathways in different experimental models both *in vitro* and *in vivo* (Meng et al., 2013). Regardless, that effect was not observed in the undifferentiated SH-SY5Y cells in our study, as no changes in ROS levels were detected after exposure for 4, 8, 12 or

24 hours to any of the concentrations assayed of MC-LR (0-37 µg/mL). In our study, however, a decrease of GSH levels was observed after 24 hours to all the concentrations of exposure assayed, which might be the cause of the lack of response in the ROS levels, as GSH could act directly against ROS (Circu and Aw, 2010). In this sense, Meng et al. (2013) also reported an oxidative stress generation by MC-LR in differentiated rat pheochromocytoma PC12 cells, detecting a significant increase of ROS levels after exposure to 5, 7.5 and 10 µM after 3 hours of exposure. Furthermore, they detected the increase in the ROS levels only after 0-6 hours of exposure, decreasing to basal levels after 18 hours. This would point out the rapid response to the cells against MC-LR.

Furthermore, CYN also has been reported to be able to cause oxidative stress as one of its main mechanisms of action (Buratti et al., 2017). Regardless, when undifferentiated SH-SY5Y cells were exposed to CYN for 4, 8, 12, and 24 hours, no changes in ROS or GSH levels were observed. Nonetheless, the exposure to CYN+ led to an increase of ROS levels after 8, 12 and 24 hours of exposure to all the concentrations assayed (0 – 1.5 µg/mL) (3.6 µM). Meanwhile, this same extract induced a significant decrease of GSH levels was detected only after exposure for 4 and 24 hours to 0.75 µg/mL (1.8 µM) CYN+. When the same experiment was performed with CYN-, an increase of ROS levels was also detected after 24h of exposure to 0.75 and 1.5 µg/mL, and after 12h of exposure to 1.5 µg/mL. Nonetheless, the increase of ROS levels by CYN- was not as big as the caused by CYN+. Taking into account that CYN caused no alteration in this parameter, this would suggest, again, the interaction between the cyanotoxin and the rest of the cyanobacterial compounds present in the extract. Concerning to GSH levels, CYN- did cause a decrease after 8 and 24 hours of exposure at all the concentrations assayed, which could also compensate ROS increment and thus, be a possible explanation for the differences when compared to CYN+. Although there are no more studies in relation to oxidative stress *in vitro* for in the nervous system, Guzmán-Guillén et al. (2015) reported an increase in LPO levels in brain after exposure to CYN+ culture in tilapia fish *in vivo*, so did Da Silva et al. (2018) in *Hoplias malabaricus*. However, it is also worth to mention that these last authors reported differences in the oxidative stress parameters after exposure to pure CYN and CYN+. These differences are in agreement to the results obtained in our undifferentiated cell model.

In addition, one of the main mechanisms of action reported for the neurotoxicity caused by CPF is the oxidative stress production (Park et al., 2013, 2015). In this sense, the ROS generation did not increase after exposure to 4, 8, 12 or 24 hours in undifferentiated cells to 0-84 µg/mL (0-239.6 µM). Nonetheless, all concentrations and times of exposure assayed led to a significant concentration-dependent decrease in the GSH levels, demonstrating a response of the cells against the oxidative stress produced by this pesticide. In agreement, Park et al. (2015) also reported an increase in the ROS levels after exposure to 50 and 100 µM (17.53 and 35.05 µg/mL)

for 24 hours in the SH-SY5Y cells. In the same cell line, Xu et al. (2017) demonstrated also an increase of ROS levels after exposure to 40 and 80 µM CPF (14.02 and 28.05 µg/mL) for 48 hours. However, these last two studies did not evaluate the GSH levels, which would help to clarify the antioxidant response of this cell line. Moreover, Giordano et al. (2007) studied the effect of CPF in regular murine cerebellar granule neurons and in the same cells lacking the modifier subunit of glutamate cysteine ligase (GCL), which is the limiting enzyme in the GSH synthesis. In this regard, these authors found that the ones without GCL presented more cytotoxic effects after exposure to CPF. Furthermore, they also reported an increase of the ROS levels after exposure to 1 µM CPF (0.35 µg/mL) after 1 hour of exposure, being this increase higher in those cells lacking GCL (Giordano et al., 2007). These results manifest the possible role of GSH in the cytotoxicity exerted by CPF.

In general, the results demonstrated that pure CYN, MC-LR or CPF do not seem to affect the ROS levels. Nonetheless, CYN+ caused an increase of this parameter after 8 up to 24 hours, so did CYN- after exposure to the highest concentrations and times of exposure. Nonetheless, CPF did cause a significant decrease of GSH levels compared to the control, so did MC-LR at all concentrations of exposure after 24 hours, CYN+ after 0.075 µg/mL, and CYN- after 8 and 24 hours, while pure CYN did not alter this parameter either. These data would suggest that oxidative stress is involved for all the toxics except for pure CYN, in the experimental model used, by decreasing the GSH levels, which could be the cause for the lack of effect observed in ROS levels.

1.3. AChE

Concerning the effects observed in AChE, MC-LR caused a significant increase in this enzymatic activity only after the highest concentrations of exposure (37 µg/mL) in the undifferentiated SH-SY5Y, while the differentiated cells did not seem to be affected at any of the concentrations assayed. Up to now, no other reports studying the AChE activity after exposure to MC-LR have been reported *in vitro*. However, *in vivo*, MC-LR has demonstrated to affect AChE levels, although this variation is not consistent, depending on the conditions of exposure and the experimental model (Wu et al., 2016; Gélinas et al., 2012; Kist et al., 2012; Qian et al.; 2018).

The studies concerning the effects on AChE caused by CYN led to a concentration-dependent decrease in this enzymatic activity after exposure to all the concentrations assayed for 24 hours. The *in vitro* studies available are very scarce. The first study reported was the one performed by Kiss et al. (2002) in *Helix pomatia* and *Lymnaea stagnalis* after exposure to a *R. raciborskii* purified fraction. These authors detected a decrease in the ACh-induced membrane response, although CYN had no direct effect in the neuronal membrane. However, Vehovszky et al. (2013) reported on central nervous system preparations of *Helix pomatia* that CYN+ did not

produce the same cholinergic inhibitory effects. With respect to the effects observed *in vivo* in fish, Guzmán-Guillén et al. (2015) detected a reduction in AChE activity after suchronical exposure to a CYN+ culture in tilapia, although that inhibition was too low to cause neurological symptoms. In contrast, Da Silva et al. (2018) detected an increase of AChE activity in brain after exposure for 7 days to CYN+, while no significant alterations were detected after the same time of exposure to purified CYN. Thus, the results concerning the effects of CYN in AChE activity are controversial and more studies are required in order to elucidate its effect in this enzymatic activity and, therefore, its possible effects on neurotransmission.

Furthermore, according to Park et al. (2013, 2015), the oxidative stress produced by CPF might be related to AChE inhibition, which is known to be the main mechanism of action for this OP, both *in vitro* and *in vivo* (Eaton et al., 2008). Indeed, the results obtained in undifferentiated SH-SY5Y exposed for 24 hours to 0-84 µg/mL (0-239.6 µM) led to a decrease in the AChE after all the concentrations of exposure. Regardless, when the same experiment was performed in the differentiated cells, no changes in the AChE were observed. In contrast, an upregulation of the AChE genes after differentiation was reported by De Medeiros et al. (2019) in the same cell line. In this line, Jameson et al. (2007) reported an increase in the AChE mRNA levels in the differentiated PC12 cells exposed to CPF, which confirms that AChE activity is, indeed, involved in the toxicity exerted in that cell line. *In vivo,* when Xu et al. (2017) measured the AChE in rats brain after oral exposure for 90 days, they also detected a dose-dependent decrease in this enzymatic activity after all the doses assayed (0- 15mg/kg/day).

To sum up, the studies led to different results for the toxics when the cells are undifferentiated versus differentiated. This difference in the response might be due to the structural and functional modifications of the cells during the differentiation process. In this sense, Attoff et al. (2020) and De Medeiros et al. (2019) have reported the variation at the gene expression levels during the differentiation process in the SH-SY5Y cells.

1.4. Morphological studies

Besides the cytotoxic effects observed, some morphological studies were performed in order to assess the damage detected in the cells. In this regard, the exposure to MC-LR in both undifferentiated and differentiated SH-SY5Y cells led to cell death shown by cytoplasm fragmentation, chromatin condensation, nucleolar segregation, endoplasmic reticulum dilatation, lipidic vacuoles and presence of heterophagosomes. In this line, Meng et al. (2011) described apoptotic effects such as the reorganization of the cytoskeleton of differentiated PC12 cells exposed to 10 µM. Furthermore, Zhang et al. (2018) reported neurite degeneration and cell death in undifferentiated SH-SY5Y cells after exposure to 10 µM MC-LR. This effect has also been

observed in primary murine cerebellar granule neurons, where Feurstein et al. (2011) reported a slight impairment of the neurite network, which agrees with the findings of Rozman et al. (2017) in primary rat astrocytes. Zhang et al. (2020) also detected apoptosis of hippocampal neuronal cells after exposure to MC-LR, exerting mainly mitochondrial damage. These results agree with the findings *in vivo*. In this sense, Li et al. (2009a) suggested damage on sensory neurons in *C. elegans* after exposure to 20-160 µg/L of a cyanobacterial extract containing MC-LR. In addition, Shin et al. (2018) and Wang et al. (2018; 2019) reported the same effects after exposure to pure MC-LR. According to some authors, a possible cause for apoptosis can be an increase in the AChE activity, which would lead to the formation of apoptosomes or affect the expression of apoptotic genes (Zhu et al., 2007), which is in agreement to the response detected in the undifferentiated SH-SY5Y in our experiment.

Concerning the exposure to CYN, the effects on the morphology demonstrate that this cyanotoxin causes clear signs of cellular death by apoptosis in both variants of the SH-SY5Y cells after exposure to 1 µg/mL (2.4 µM) and 0.3 µg/mL (0.7 µM) CYN in undifferentiated and differentiated cells, respectively. This is in agreement to the findings obtained by Takser et al. (2016), who found a significant increase in the apoptotic caspases 3/7 activity after exposure to 10 µM CYN in RAW264.7, BV-2 and N2a cells, being the effect more prominent in the last two cell lines. Furthermore, these authors also observed a significant increase in the pro-inflammatory response to 10 µM in BV-2 and N2a cells, demonstrating that low CYN concentrations are highly relevant for neurodegeneration processes. These effects were also corroborated *in vivo* by Kinnear et al. (2007), Guzmán-Guillén et al. (2015), Da Silva et al. (2018) and Rabelo et al. (2021), who demonstrated the presence of CYN in brains after exposure to *Bufo marinus* tadpoles and three different species of fish, respectively, detecting histopathological changes in the brain and necrosis. Thus, although the main mechanism of neurotoxicity for CYN is not well-known yet, the authors suggest that oxidative stress or AChE activity might be involved (Pichardo et al., 2017; Rabelo et al., 2021).

Regarding the pesticide, the effects in the AChE and in GSH levels are related to mitochondrial dysfunction and, consequently, to apoptosis, according to some authors (De Oliveira et al., 2016; Park et al., 2013). In this sense, CPF caused mitochondrial alterations that led to apoptosis in both undifferentiated and differentiated SH-SY5Y cells, which agrees with the results obtained by Park et al. (2015) in the same cell line, who reported the activation of caspase-9 and -3, and an increase of cytosolic cytochrome c after exposure to CPF. The activation in the caspase-3 by downregulation of Bcl-2 and Bcl-xL, was also reported by Raszweski et al. (2015) in the undifferentiated SH-SY5Y cells, demonstrating to be a possible pathway for apoptotic processes.

Therefore, the morphological studies concluded that all of the toxics cause signs of apoptosis at almost all the concentrations of exposure assayed in both undifferentiated and differentiated cells.

1.5. Synapses

As CYN demonstrated to exert some effects at a synaptic level due to the effects observed on the AChE, more synaptic studies were perfomed, this time in a more evolved experimental model, neuronal cultures from primary hippocampus from E17 embrionic CD1 mice. In these neurons, CYN led to a decrease in cell viability measured using NeuN and MAP2 biomarkers, concentration and time-dependently, by causing a significant decrease after exposure to 0.5 and 1 µg/mL after 12 hours of exposure, and to all the concentrations assayed after 24 and 48 hours, obtaining almost a decrease of the viability up to less than 20% after 48 hours exposure to all the concentrations assayed. According to these results, when the biomarkers for synaptophysin and PSD95 were studied, after 12 hours of exposure, a decrease of the number of synapses was observed after the exposure to 0-1 µg/mL concentration-dependently compared to the negative control. This effect is also seen after the exposure for 24 hours in all the concentrations of exposure. However, when the experiment was performed for 48 hours of exposure, no synapses were detected, as was expected taking into account the effects observed in cell viability. Our results showed, for the first time, that CYN produces a decrease in neuronal synaptic function.

1.6. NAChRs

Concerning the effects on the synapses in 3-days differentiated SH-SY5Y cells, some experiments were performed in order to see if CYN and CPF affected the nAChRs. The activation of these receptors leads to cell depolarization and the increase of the intracellular free calcium (Loser et al., 2021). These receptors are expressed in the central nervous system, and their affectation during development could cause disturbances of neurotransmitter signaling development, leading to altered brain connectivity in later life (Grandjean and Landrigan, 2006; 2014). In this sense, when the cells were acutely exposed to CYN, no agonistic or antagonistic effects were observed after exposure to 0-1 µg/mL (2.4 µM) in the presence of PNU, which is an allosteric modulator of the α7 isoform of this receptor. This is, to our knowledge, the first time that CYN is studied as a developmental neurotoxicant.

Nnonetheless, the preexposure to 0-100 µg/mL (285.23 µM) of CPF and then acute exposure to 11.1 µM nicotine led to a decrease in the response of these receptors to nicotine after preexposure to the highest concentrations. The studies performed on the effects in AChRs are

scarce. It has been reported that CPF can produce different regional patters of change in the density of different mAChRs (Chaudhuri et al., 1993). Regarding, Huff et al. (1994) reported a reversible binding of CPF-oxon to m2 muscarinic receptor that would not interfere with the interaction of this receptor with its agonist carbachol in rat striatum. This effect was observed after exposure to concentrations that caused a minimum change in the AChE levels. Furthermore, the mAChRs antagonist atropine did not cause any changes in the response either. These authors did not elucidate the effects of the interaction OP-mAChRs, however, they reported that it was independent of AChE inhibition (Huff et al., 1994). In addition, Olivier et al. (2001) also observed a reduction of cAMP formation in cortical slices of 7, 21 and 90 day-old rats after exposure to CPF-oxon, partially caused by its interaction with mAChRs. This cAMP is a primary modulator of cell differentiation, among many other physiological processes (Slotkin, 2006).

1.7. Cell viability and neurite outgrowth

In addition to the calcium measurements to detect possible effects on developmental neurotoxicity, 6 days-differentiated SH-SY5Y cells were exposed during differerentiation to 0-1 µg/mL (2.4 µM), providing an EC_{50} value of 0.2 µg/mL (0.49 µM) after the resazurin assay for viability. Then, the changes on the neurite outgrowth were assayed using a concentration range of 0-0.2 µg/mL (0.49 µM) in order to assess that the effect observed is due to the effects of CYN in the neurite outgrowth *per sé* and is not because of the viability reduction. Our results led to a concentration-dependent decrease of the neurite outgrowth. Thus, this is the first time that CYN is reported to be able to cause developmental neurotoxicity.

Besides its already known effects on neurotoxicity, CPF has also demonstrated to exert developmental neurotoxicity, as is able to cross the placenta (Burke et al., 2017). Despite being considered the AChE inhibition its main mechanism of action, many studies report non-ChE-associated mechanisms as well (Abreu-Villaca and Levin, 2017; Rahman et al., 2021). In this sense, in our study, 6 days-differentiated SH-SY5Y cells were exposed to 0-200 µg/mL (570.47 µM) during differentiation demonstrated to be more sensitive than the undifferentiated cells, providing an EC_{50} value of 63.84 µg/mL (182.1 µM). Thus, we assayed the changes on the neurite outgrowth of this cell model using a concentration range of 0-64 µg/mL (182.56 µM) in order to assess that the effect observed is due to the effects of CPF in the neurite outgrowth *per sé* and is not produced because of the viability reduction. This experiment led to a concentration-dependent decrease of the neurite outgrowth. In this line, Yang et al. (2008) also reported an inhibition of the axonal outgrowth of primary cultures of embryonic rat dorsal root ganglia neurons after exposure for 24 hours to CPF in all the concentrations assayed (0.001 – 10 µM), not detecting significant changes in the number of axons per neuron, but in the length. In addition, these authors

reported that this effect seems to be linked to the AChE activity, but not to its inhibition. Furthermore, *in vivo,* CPF has demonstrated to cause changes in downstream signaling involving neurotrophins in rats, which regulates the neurite outgrowth, cell differentiation and neuronal repair (Slotkin et al., 2007; Betancourt and Carr, 2004; Burke et al., 2017).

2. Combinational studies

As cyanotoxins are likely to appear together and in combination with some other toxicants such as pesticides (Metcalf and Codd, 2020), the combination of CYN + MC-LR and CYN + CPF were assayed.

2.1. Cell viability

Concerning the CYN + MC-LR combination, the ratios used were 1:37 and 0.3:45, for undifferentiated and differentiated cells, respectively, according to the values previously obtained for isolated cytotoxicity. In both undifferentiated and differentiated cells, the combination demonstrated to exert different cytotoxicity than the compounds isolated, being more similar the response to the combination to the one obtained after exposure to MC-LR alone. Thus, in order to study the cyototoxicity exerted by the combination, the type of interaction of these two compounds in the SH-SY5Y was studied using the isobologram method. Our results showed that the combination would cause antagonism after exposure to low concentrations but additive effects after exposure to high concentrations after 24 and 48 hours of exposure in the undifferentiated cells. However, in the differentiated cells, an antagonistic response was obtained after all times and concentrations of exposure. The response in the differentiated cells agrees to the one obtained by Gutiérrez-Praena et al. (2018), who also obtained an antagonistic response after all the concentrations for CYN + MC-LR combination after 24 and 48 hours in HepG2 cells exposed to a ratio of 1:22.5 CYN:MC-LR. In addition, CYN + MC-LR led to a synergistic response in the growth rate of *Chlorella vulgaris* (Pinheiro et al., 2016). Furthermore, despite not studying the type of combinatorial response, Takser et al. (2016) also studied the combination of CYN, MC-LR and anatoxin-a in an equimolar concentration (3.33 µM), obtaining a significant reduction of cell viability in RAW264.7 cells after 24, 48 and 72 hours of exposure. In addition, the combination caused total cell death in both RAW264.7 and BV-2 cell lines (Takser et al., 2016). According to the authors, the combination was more toxic than the response obtained after exposure to the isolated cyanotoxins

When the cells were exposed to the combination of CYN + CPF, the results were varied between undifferentiated and differentiated cells. An antagonistic response was obtained in the

undifferentiated cells after 24 and 48 hours of exposure except for the lowest concentrations after 48 hours, that led to synergism. This response is the same as the one observed in the differentiated cells after 48 hours. Regardless, when differentiated cells were exposed to the combination for 24 hours, the highest concentrations led to synergism. Thus, a possible explanation might be the different concentrations ratio of exposure which were selected based in previous viability assays being the ratios were 1:84 in undifferentiated and 0.3:34 in differentiated for CYN:CPF. In addition, when the combination of CYN + CPF was exposed during 6 days of differentiation in SH-SY5Y cells, the response obtained was antagonism. This method has also been used to assess the effects of toxins mixtures by Juan-García et al. (2016) in mycotoxins, detecting also changes after 24, 48 and 72 hours of exposure, by obtaining synergism, additivity and antagonism, respectively in HepG2 cells. Although the combinatorial studies concerning CYN + CPF are scarce, the effects of the mixture of cyanotoxins and some other pollutants has been reported. Consequently, Wang et al. (2017) observed also synergism after low concentrations and antagonism after exposure to higher concentrations of the cyanotoxin MC-LR and Cu in the aquatic plant *Vallisneria natans*. The studies performed *in vivo* point out an additive effects in *Daphnia pulicaria* after exposure to a strain of *M. aeruginosa* and the pesticide carbaryl for 24 hours, but caused synergism in body deformations of newborns. On the contrary, Asselman et al. (2013) observed antagonism or no interaction of carbaryl and four different cyanobacterial species (*Aphanizomenon* sp., *R. raciborskii, M. aeruginosa* and *Oscillatoria* sp.) in *Daphnia pulex*. In addition, same authors studied the effects of *M. aeruginosa* combined with four different pesticides, including CPF, after 21 days of exposure. In this case, the authors reported an additive response instead, despite presenting the same mechanism of action than carbaryl (Asselman et al., 2014). This highlight the importance of not generalizing the interactive effects of compounds with the same pathway (Asselman et al., 2014). In bigger animals, Ondracek et al. (2012) exposed Japanese quails to a cyanobacterial biomass-combination, paraoxon and the anticoagulant bromadilone obtaining more severe damage in these organisms after exposure to the combination compared to the effects observed by the compounds on their own.

2.2.   Oxidative stress

Concerning oxidative stress damage, no alterations were observed in the undifferentiated SH-SY5Y cells after exposure to any of the concentrations tested of CYN + MC-LR for neither of the times of exposure in ROS levels, in agreement to the results obtained after exposure to the cyanotoxins alone. However, a slight decrease in GSH levels was observed after exposure to the lowest concentrations for 8 hours and the lowest one after 4 hours. This decrease is in agreement

to the results observed after exposure to MC-LR alone, although not in the same concentrations or times of exposure.

In relation to the oxidative stress generation of the CYN + CPF combination, the results obtained are similar to effects produced by CPF itself. No increase in the ROS levels was detected after any of the times and concentrations of exposure assayed, while the combination did cause a decrease in the GSH levels after all the concentrations and times of exposure, as observed after exposure to CPF alone. In both trials of combination of CYN with another substance (MC-LR or CPF), the results show that CYN is not the one that determines the final response. Again, this response could be produced as a compensation for the oxidative stress produced after the exposure to the toxics, which could be the reason for the unaltered ROS levels. In this cell line, Xu et al. (2017) also studied the effect of CPF in combination with the heavy metal cadmium, which demonstrated to cause oxidative stress when they were used isolated. Nonetheless, the combination did not lead to higher toxicity. Thus, the authors described a potential antagonistic interaction in oxidative stress parameters.

2.3. AChE

Furthermore, no significant changes were detected in the AChE activity after 24 hours of exposure to the CYN + MC-LR combination in the undifferentiated cells. Nonetheless, the exposure to the highest concentrations led to a significant increase in AChE activity in the differentiated SH-SY5Y. This response did not happen when the cells were exposed to the cyanotoxins alone, which could indicate that the toxic response to the mixtures is unpredictable and may not follow the pattern of any of the toxic composing it.

When the AChE was measured after exposure to the combination (CYN + CPF), they caused a decrease in this enzymatic activity in the undifferentiated cells, being that response similar to the one obtained after exposure to CPF alone. This agrees to the results obtained by Xu et al. (2017) after exposure to CPF and cadmium, as cadmium demonstrated not to exert any effects in the AChE inhibition when combined with CPF. Thus, these data would suggest that CPF is the compound determining the effects on the AChE activity. However, when the same experiment was performed in the differentiated SH-SY5Y cells, no response was observed after any of the concentrations of exposure tested.

2.4. Morphological studies

In order to see the type of damage in the cells, some morphological studies were performed in the undifferentiated and differentiated cells after exposure to the combination of CYN + MC-LR. In this sense, also apoptotic features were detected. Nonetheless, as the effect of the combination did not lead to a response as intense as expected by the toxicity of the chemicals isolated, they might have antagonistic effects. This is in agreement to the results obtained by Gutiérrez-Praena et al. (2018) in the hepatic cell line HepG2, who also reported an antagonistic response in the cells exposed to CYN + MC-LR.

Concerning the combinational studies for CYN + CPF, the mixture led to a more severe cell death by apoptosis compared to the response obtained after exposure to the toxics alone in both undifferentiated and differentiated cells, which is in agreement to the responses obtained by Raszewski et al. (2015) and Xu et al. (2017) in the experiments mentioned above.

These results show that the toxic effect follows the same pattern regardless the differentiation state of the cell experimental model. In addition, it is important to take into account the type of substances that form this combination, since in the case of MC-LR the effect is less compared to CPF.

2.5. Neurite outgrowth

The studies on neurite outgrowth for the combination of CYN + CPF led to a decrease of the neurite outgrowth significant only after the highest concentrations assayed (0.07 µg/mL CYN and 24.17 µg/mL CPF). However, when the cells were exposed to those concentrations isolated, the effect observed was higher than the one obtained by the combination, which would demonstrate the antagonistic effects observed in our cells.

V. CONCLUSIONS

Conclusions

Taking into account all the findings explained above, the following conclusions were obtained through the development of the present Doctoral Thesis:

FIRST. The review from the literature concerning the neurotoxic effects of MCs and CYN indicates these cyanotoxins have demonstrated some effects in the nervous system both *in vitro* and *in vivo*. In this sense, MC-LR has demonstrated to be the most studied congener, being able to cross the blood-brain-barrier and causing histopathological changes in brain, deregulation of biochemical parameters such as oxidative stress and inhibition of protein phosphatases, and behavioral alterations. However, the studies concerning the neurotoxicity exerted by CYN are very scarce, leading mainly to apoptosis, oxidative stress, and alteration of AChE.

SECOND. Both MC-LR and CYN caused a concentration-dependent cytotoxicity, isolated and in combination, both in undifferentiated and differentiated SH-SY5Y cells. This was corroborated by the results obtained in the morphological studies, where mainly apoptotic signs were found in a concentration-dependent manner. The combination of these cyanotoxins led to antagonism in the experimental models used. Only MC-LR and the combination led to oxidative stress in the undifferentiated SH-SY5Y cells. The AChE activity was reduced only after exposure to CYN in the differentiated cells.

THIRD. The combination of CYN and CPF led to oxidative stress, AChE activity inhibition, and apoptosis in the undifferentiated SH-SY5Y cells, similar to the response obtained after exposure to CPF itself. The combination led to an antagonistic response, although low concentrations after 48 hours of exposure led to synergism.

FORTH. The combination of CYN and CPF caused a concentration-dependent decrease of viability in the differentiated SH-SY5Y, so did CPF alone, which is in agreement with the apoptosis observed after the morphological studies. The isobologram method detected antagonism after 24 hours of exposure to the lowest concentrations, and synergism at the highest, which was contrary to the response obtained after 48 hours. No changes in AChE were detected for the combination.

FIFTH. The exposure to CYN+ and, to lesser extent, to CYN-, caused cytotoxicity in the undifferentiated SH-SY5Y cells, demonstrating that the effects observed are caused by CYN. CYN+ also led to an increase in ROS with no alteration of GSH, while CYN- caused a less significant rise of ROS levels but a strong alteration of GSH levels, demonstrating that other compounds from the extract could be the main cause for this outcome.

SIXTH. The exposure of murine primary cultures to pure CYN led to a concentration- and time-dependent decrease in neuronal viability. In addition, CYN was also demonstrated to

cause a decrease of the number of synapses confirming, for the first time, an involvement of the neuronal function by CYN *in vitro*.

SEVENTH. CYN demonstrated not to cause any acute effects in nAChRs in SH-SY5Y cells after 3 days of differentiation. However, CPF was demonstrated to act as an antagonist for this receptor when exposed acutely. In addition, both toxics isolated and in combination led to a concentration-dependent decrease of cell viability when exposed during differentiation for 6 days in SH-SY5Y cells. Furthermore, the response obtained by the combination was antagonistic. Concerning the effects on neurite outgrowth, both toxics isolated and in combination demonstrated to cause a decrease in this parameter, which indicates their capacity to exert some developmental neurotoxic effects.

GENERAL CONCLUSION. The results obtained in this doctoral thesis indicate that CYN is able to cause neurotoxicity, so are MC-LR and CPF. Furthermore, these data highlight the importance to study the toxics, not only isolated but also in combination, as their toxic features might vary depending on the environment. More research studies focused on the effects of these cyanotoxins on the nervous system are necessary to understand the role they play in the development of neurodegenerative diseases. In addition, more studies concerning the combinational effects of these cyanotoxins in a more realistic scenario are required to fully assess the toxicity of these compounds.

VI.   REFERENCES

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